IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Rosenblum, M. G.	§	ART UNIT:
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FILED: February 19, 1998	§	
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SERIAL NO.: 09/026,882	§	S. Huff
	§	
FOR: Immunotoxins Directed Against CD33 Related Surface Antigens	§	DOCKET: D5442C/CIP

Commissioner of Patents and Trademarks

Washington, D.C. 20231

ATTENTION: Board of Patent Appeals and Interferences

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on September 13, 2002. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is the Research Development Foundation, the Assignee, as evidenced by an Assignment recorded in the Patent and Trademark Office at Reel 012024, Frame 0723 on July 24, 2001.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

Originally claims 1-17 were filed with this Application and subject to restriction. In response to the Restriction Requirement, claims 8-15 were elected and claims 1-7 and 16-17 were withdrawn from consideration, but not canceled. In response

to an Office Action mailed December 27, 1999, Applicant amended claims 8 and 12. Therefore, claims 8-15 are pending of which claim 8 is an independent claim.

IV. STATUS OF AMENDMENTS

Subsequent to an Office Action mailed December 27, 1999, Applicants submitted a Response which amended claims 8 and 12. No further claim amendments have been submitted up to and including the mailing of a Final Rejection on July 5, 2002 for which Final Rejection a Notice of Appeal was filed. All pending claims are shown in Appendix A.

V. SUMMARY OF THE INVENTION

The present invention is directed to a method of treating a neoplastic cell via administration to the cell of an effective dose of a conjugate of a protein with binding specificity for an antigen domain of CD33 and a recombinant gelonin or a functionally active

recombinant gelonin fragment (page 4, ll. 6-15). Preferably, such neoplastic cells are acute and chronic myeloid leukemias and lymphoid leukemias (pg. 3, ll. 23). The neoplastic cell may be located *in vitro* (pg. 31, Ex. 10), *in vivo* (pg. 37, Ex. 14) or in bone marrow (pg. 35, Ex. 13). The neoplastic cell may be in a human host or in a non-human host. (pg. 18, ll. 10-11). The composition when used in the treatment method retards the rate of growth of the neoplastic cell. Further, if the neoplastic cell is in a host, the growth of the neoplastic cell in the host is inhibited and the survival time of the host is extended. (pg. 37, ll. 20-21)

Specifically, the present invention is useful because it provides a method of treating a neoplastic cell with a composition that overcomes the problems associated with immunotoxins at the time of the instant invention. The composition functions as an immunotoxin to selectively kill tumor cells characterized by the expression of the CD33 antigenic protein such as acute non-lymphoid leukemic cells and acute myelogenous leukemic cells while. Thus, the immunotoxin i) has suitable tumor-specific targets that are not found on vital non-tumoral cells; ii) does not lose toxin potency or mAb activity after conjugation; iii) does not cause unwanted cytotoxicity to nontarget cells and tissues resulting from nonspecific

internalization of the immunotoxin; iv) induce an immunogenic response; and v) has the pharmacological ability to target tumor sites adequately (pg. 3, ll. 13-20).

VI. ISSUES

35 U.S.C. §103 rejections

Whether claims 8-15 are obvious under 35 U.S.C. §103 over **Tanimoto** *et al.* (*Leukemia*, vol. 3, p. 339 (1989)) or **Scheinberg** (*Leukemia*, vol. 3, p. 440 (1989)) or **Scheinberg** (U.S. 5,730,982) in view of **Thorpe** *et al.* (*Immunological Reviews*, Vol. 62, p. 119 (1982)) and **Andrews** *et al.* (*Blood*, Vol. 62, p. 124 (1983) and **Rosenblum** *et al.* (U.S. 5,631,348).

VII. GROUPING OF CLAIMS

The rejected claims stand or fall together.

VIII. ARGUMENTS

Rejection Under 35 U.S.C. §103

In the Final Action mailed July 5, 2002 in response to a Request for Continued Examination filed May 15, 2002, the Examiner maintained the rejection of pending claims 8-15 (for substantially the same reasons of record in paper no. 7, mailed December 27, 1999) as being obvious over the prior art cited under 35 U.S.C. §103 in Section VI Issues *supra*. Applicants vigorously traverse this rejection.

In the Office Action mailed December 27, 1999 and in subsequent actions, the Examiner maintained that the prior art provided sufficient motivation to construct Applicant's antibody conjugates. The Examiner stated that **Tanimoto** *et al.*, **Scheinberg** *et al.* (Leukemia) and **Scheinberg** (U.S. 5,730,982) each teach the monoclonal antibody M195 specific to CD33 protein. Furthermore, the Examiner indicated **Tanimoto** *et al.* (pg. 347) and **Scheinberg** *et al.* (pg. 444) teach the potential of using M195 as a therapeutic agent as a carrier of toxins or alpha-emitting isotopes for the treatment of acute non-lymphocytic leukemia. With regard to **Scheinberg**, the

Examiner states that the reference teaches the use of M195 in a conjugate with a cytotoxic agent which can be a toxin to treat acute or chronic leukemia (col. 6, ll. 9-13, 17-29).

Additionally, **Thorpe** *et al.* teach conjugation of antibodies to toxic moieties including gelonin for effective cell killing which would have potential in therapeutic applications (pg. 147-150). **Andrews** *et al.* teach that monoclonal antibodies reactive with CD33 may be useful for the treatment of leukemia for *in vivo* or *in vitro* use. Finally, **Rosenblum** *et al.* shows that gelonin can be made recombinantly and active fragments can be isolated.

As such, the Examiner contends that the motivation to combine these references to use the antibody conjugates to treat neoplastic cells *in vitro* or *in vivo* comes from the primary references **Tanimoto** *et al.*, **Scheinberg** *et al.* and **Scheinberg** (pg. 347, pg. 444, col. 6, respectively) which

> "...clearly suggest the use of conjugates of M195 and toxin to treat leukemia. The use of gelonin as the toxin would have be obvious in view of **Thorpe** *et al*, who shows that gelonin is very effective for killing cells. The use of recombinant gelonin or active fragments within the

purview of one skilled in the art especially in view of **Rosenblum** *et al.*" (pg. 6 and pg. 8 of paper no. 7)

Applicant does not dispute that an M195 monoclonal antibody specific for CD33 protein was known in the art nor that construction of monoclonal antibodies conjugated to gelonin was known in the art at the time of the instant invention. However, Applicants strongly dispute the Examiner's contention that a person with ordinary skill in this art would have been motivated to treat neoplastic cells with an antibody conjugate comprising a mAb specific for CD33 and either recombinant gelonin or active recombinant fragments because of a **clear suggestion** (Applicant's emphasis) in the primary references that such a construct is useful for treatment of leukemia. On page 347 of *Leukemia*, Vol. 3, No. 5 (1989) **Tanimoto** *et al.* state

> "M195 may be used in this application, but its demonstrated lack of cytotoxicity in the presence of human complement or PBMC in vitro might require that the mAb carry a cytotoxic isotope or toxin to be effective. Since the antigen and antibody are rapidly internalized, this therapeutic modality **may be feasible and**

investigations of this application are underway." (Applicant's emphasis).

Also, on page 444 of *Leukemia*, Vol. 3, No. 5 (1989) **Scheinberg** *et al.* state:

> "The M195 antigen is not expressed on adult human tissues. Therefore, in addition to its use as a diagnostic marker of ANLL and as a purging agent, M195 can potentially be used as a therapeutic agent in vivo. Since the antibody does not have in vitro cytotoxic effects alsone or in the presence of human serum as a complement source, it is not likely to cause lysis of ANLL cells. However, upon binding of mAb M195, the antibody is rapidly internalized (to be published elsewhere), and thus **the application of mAb M195 as a carrier of toxins or alpha-emitting isotopes to ANLL cells *in vivo* may be feasible.**" (Applicant's emphasis)

Applicant strongly contends that neither of these statements is a clear suggestion that conjugates of M195 and toxin are used to treat leukemia. These are merely statements of intent to

investigate the possibility with no suggestion that such attempts will be successful. Although citing **Andrews** *et al.* as disclosing that monoclonal antibodies reactive with CD33 may be useful for the *in vivo* or *in vitro* treatment of leukemia, the Examiner does not include this reference when discussing the motivation to combine the prior art. However, **Andrews** *et al.* also state that

> Monoclonal antibodies reactive with differentiation antigens expressed by normal and malignant myeloid cells may be useful for treatment of nonlymphocytic leukemias, either for in vitro deletion of malignant cells from bone marrow in autologous bone marrow transplantation or for in vivo use as adjuvant therapy when tumor burden is at a minimum. The extent to which such antibodies are capable of distinguishing between leukemic cells and normal stem cells required for reconstitution of the bone marrow will determine their utility for purposes of therapy in ANL, **and thus, at present, their usefulness remains an open question**. (Applicant's emphasis)

Again, this is a suggestion that one needs to determine if the mAb's even have therapeutic efficacy and not a suggestion to use them as such.

The investigations to which **Tanimoto** *et al.* refer on page 347 are disclosed in **Scheinberg** (U.S. 5,730,982) together with the teachings in **Scheinberg** *et al.* including the internalization of M195 data indicated to be published elsewhere on page 444. Despite the disclosure in **Scheinberg** to a therapeutic agent for treatment of chronic or acute leukemia comprising M195 and a cytotoxic agent (col. 6, ll. 9-12) such as a radioisotope or a polypeptide such as a toxin or a drug or claim 1 drawn to M195 conjugated to a polypeptide toxin and claims 12 and 13 drawn to such therapeutic agents or methods of treatment using said therapeutic agent, **Scheinberg** teaches only a radioisotope conjugated to M195. Not a single teaching or enabling disclosure nor even an identified toxin other than a radioisotope is found in **Scheinberg** for any M195 antibody conjugate other than a radionuclide conjugate.

The Examiner states that **Scheinberg** teaches that the cytotoxic agent can be a toxin such as a radioisotope or a polypeptide such as a toxin or a drug (col. 6, ll. 9-13). However, the polypeptide

comprising a toxin or a drug is part of a fusion protein with the recombinant polypeptide comprising that part of M195 substantially the same as its hypervariable region. Thus, Applicants submit that **Scheinberg** teaches a radiolabeled M195 antibody or an M195-radioisotope conjugate and the use of said in treating leukemia. This does not provide motivation to one of ordinary skill in the art to combine this teaching with the other references cited *supra* to arrive at a method of treating a neoplastic cell with a CD33-specific mAb-recombinant gelonin conjugate.

Strengthening this argument is the teaching in **Thorpe** *et al.* of conjugating native gelonin to an anti-$Thy_{1.1}$ monoclonal antibody with SPDP. **Thorpe** *et al.* state that about 90% of the gelonin in the conjugates lost activity after reduction in comparison to native gelonin, even though the conjugate possessed cytotoxic effects. Conjugation of gelonin using a linker which is not susceptible to cleavage by reduction yielded conjugates of four molecular weight ranges in which the gelonin only retained 3-4% of the capacity of native gelonin to inhibit protein synthesis. The four conjugates were also 20 times less potent than those conjugated via SPDP against $Thy_{1.1}$-expressing AKR cells (pg. 147-149).

Therefore, although **Thorpe** *et al.* demonstrated that an anti-$Thy_{1.1}$ monoclonal antibody conjugate of gelonin does possess cytotoxic activity against AKR lymphoma cells or T-lymphoblasts, cytotoxicity appears to be dependent on, at least, the manner of conjugating the gelonin to the antibody. The retention of some cytotoxic activity does not preclude the possibility that some linkages will completely inactivate the gelonin. **Thorpe** *et al.* do not teach any other cell-specific monoclonal antibody-gelonin conjugates nor suggest that any others would be effective therapeutic agents, but rather suggest that:

> "In view of the variation in effectiveness of conjugates prepared by different methods there is much scope for examining further the influence of the linkage upon the stability and cytotoxic performance of the conjugate." (pg. 152, last sentence)

Further, the instant invention is drawn to using a recombinant gelonin or active recombinant fragment in the antibody conjugate. **Thorpe** *et al.* chose gelonin because of its potency in

native form, yet conjugating the toxin to an antibody appeared to significantly affect activity of the conjugated gelonin. **Rosenblum** *et al.* cite, *inter alia*, **Thorpe** *et al.* and corroborate these difficulties encountered by **Thorpe** *et al.* and others in using gelonin stating that chemical modification of gelonin and cellular targeting moieties can reduce targeting efficiency and cytotoxic potential of gelonin itself (col. 2, ll. 15-34, 57-63). **Rosenblum** *et al.* further state that the ability to produce synthetic gelonin toxin through recombinant technology provides a reproducible source of the toxin (col. 2, ll. 65-67).

The Examiner states that **Rosenblum** *et al.* show that gelonin can be made recombinantly and active fragments can be isolated. **Rosenblum** *et al.* teach a substantially pure gelonin having a specific amino acid sequence and a degenerate DNA sequence. As recombinant technology is known and standard, **Rosenblum** *et al.* only suggest basic ways in which to produce recombinant gelonin molecules. The degenerate DNA sequence was determined from the amino acid sequence of gelonin isolated and purified from *Gelonium multiflorum*, however, the actual nucleotide sequence of the isolated

protein had not been determined, no cDNA was cloned and thus, no recombinant gelonin produced.

As a recombinant gelonin or recombinant fragment thereof is a synthetic biomolecule and may not appear in nature, one can not necessarily predict how it will function absent some prior knowledge correlating structure to function. **Rosenblum** *et al.* suggest that the native gelonin protein be compared to similar proteins to form active recombinant molecules. Yet this is only a motivation to try various manipulations and not a suggestion or teaching that such manipulation would be successful and would not require undue experimentation. Furthermore, even if an active recombinant gelonin is produced, one of ordinary skill in the art at the time of the invention, in light of the problems with chemically modifying gelonin as disclosed in **Thorpe** *et al.* and **Rosenblum** *et al.*, would not be sure that the recombinant gelonin would be active either before or after linking to an antibody.

Thus Applicant submits that one of ordinary skill in the art would not be motivated to combine the primary references of **Tanimoto** *et al.*, **Scheinberg** *et al.* or **Scheinberg** with **Andrews** *et al.*, **Thorpe** *et al.* and **Rosenblum** *et al.* Even if, *arguendo*, these

references taught all the elements of the instant invention, one of ordinary skill in the art at best only would be motivated to try various M195 monoclonal antibody-recombinant gelonin or M195 monoclonal antibody-recombinant gelonin fragment conjugates to treat an acute or chronic leukemic cell. However, "obvious to try" is not the standard of 35 U.S.C. §103. Additionally, no suggestion is found of a reasonable expectation of success particularly in that the primary references disclose that no attempt has yet been made to examine either a therapeutic construct or a therapeutic treatment using an M195-toxin construct.

Respectfully, Applicant submits that the Examiner can not use hindsight to state that one of ordinary skill in the art, in light of the broad statements in the prior art directed to things that should be done in order to further the art, would glean a suggestion to or be motivated to treat a neoplastic cell with Applicant's CD33-specific monoclonal-antibody conjugate. Thus, it was improper to combine the references. The Patent and Trademark Office has a burden to establish a *prima facie* case of obviousness under 35 U.S.C. §103. Since the combination of cited references does not teach the instant invention as recited in amended independent claim 8, Applicants

assert that the PTO has failed to provide a *prima facie* case of obviousness. For the reasons given above, Applicants respectfully urge that the decision of the Examiner should be reversed and that claims 8-15 be allowed.

Respectfully submitted,

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CLAIMS ON APPEAL

8. (amended) A method of treating a neoplastic cell comprising administering to said cell a therapeutically effective dose of a composition comprising a conjugate of a protein exhibiting binding specificity for an antigen domain of CD33 protein and a gelonin toxin selected from the group consisting of recombinant gelonin and functionally active recombinant gelonin fragments.

9. The method of claim 8, wherein said cell is selected from the group consisting of acute and chronic myeloid leukemias, acute and chronic myelodysplastic syndromes, refractory anemias, lymphoid leukemias and undifferentiated leukemias.

10. The method of claim 8, wherein said composition retards the rate of growth of said cells.

11. The method of claim 8, wherein said neoplastic cell is in a human or non-human.

12. (amended) The method of claim 8, wherein said composition inhibits the growth of said neoplastic cell in a host.

13. The method of claim 8, wherein said composition extends the survival time of a host of said neoplastic cell.

14. The method of claim 8, wherein said neoplastic cell is in vitro.

15. The method of claim 8, wherein said neoplastic cell is in bone marrow.

Restricted Expression of an Early Myeloid and Monocytic Cell Surface Antigen Defined by Monoclonal Antibody M195

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A mouse monoclonal IgG2a antibody, M195, with reactivity restricted to early myeloid cells, acute non-lymphoid leukemia cells (ANLL), and monocytic cells is described. The antibody was derived from a mouse immunized with live human leukemic myeloblasts. Specificity of binding of mAb M195 was determined by protein-A red blood cell rosetting assays, immunoabsorption, radioimmunoassays with Iodine-125 labeled M195 IgG and F(Ab)'2, and complement cytotoxicity with live human cells and cell lines representing a broad range of lineages and tissues. Antigen expression was restricted to myeloid and monocytic leukemia cell lines and a fraction of mature adherent monocytes. Mature myeloid cells, T and B cells, erythrocytes, and platelets were negative. The antigen was not expressed on adult human tissues in immunoperoxidase and immunofluorescence assays. Blocking antigen was not found in the serum of patients with ANLL. Ten thousand sites per cell were expressed on myeloid or monocytic leukemia cell lines and 5000 sites per cell on mature monocytes. M195 IgG bound to its antigen target with an avidity of 3 × 10^9 liters/mol and induced rapid modulation of the antigen. M195 IgG was able to effectively kill cells with rabbit or guinea pig complement, but not human complement. The antibody did not mediate antibody dependent cellular cytotoxicity. The molecular nature of the target antigen remains unknown but it appears to be carried on the CD33 protein p67. Because of its restricted distribution on myelomonocytic cells, mAb M195 may be useful in studying myeloid differentiation, in the clinical diagnosis of ANLL, in purging of bone marrow of ANLL, and/or in monoclonal antibody therapy in vivo.

INTRODUCTION

THE ANTIGENS displayed on the surface of acute nonlymphocytic leukemia (ANLL) cells and hematopoietic progenitor cells are being mapped in a number of laboratories using monoclonal antibodies (mAbs) (1). These studies have been directed at identifying antigens that are useful in distinguishing lymphoid from nonlymphoid leukemias (2–4), in subtyping of acute myelogenous leukemia, and in predicting outcome (5–10) and in therapy in vivo (11) or via bone marrow purging ex vivo (12). Antigens defining ANLL cells also identify normal hematopoietic cells during early stages of their development and thus should be classified as differentiation antigens rather than leukemia specific antigens.

Antigens restricted to the earliest stages of hematopoietic development are of particular interest since ANLL is thought to be derived from these cells (13–16). mAbs identifying these early cells can help in their purification or the study of growth regulation and control of differentiation (17). Such early progenitors may be useful for autologous reinfusion in bone marrow rescue (18). Studies of bone marrow from patients with ANLL have shown that the clonogenic cells are probably derived from a subset of cells which are phenotypically more immature than the majority of cells in circulation (14, 15). This suggests that analysis of the development of leukemia cells, as well as therapeutic trials, should also be directed at these early cells and not simply the phenotypically predominant cells in the marrow and peripheral blood.

Several mAbs restricted to hematopoietic progenitors have been described: monoclonal antibodies MY10, 3C5, and 12.8 recognize a 115-kDa glycoprotein (gp115 [CD34]) found on normal colony forming cells, myeloblasts, and leukemic blasts from most patients with ANLL and acute lymphoid leukemias (19–21). mAb NHL-30.5 identifies a 180-kDa protein found on a similar distribution of cells (22, 23). My9 and L4F3 antibodies identify a 67-kDa glycoprotein (CD33) (24–27) which is expressed on slightly more mature progenitors (subsets of CFU-GEMM and some older cells) and is restricted to leukemias of the myeloid and monocytic lineage. Long-term culture studies suggest that elimination of cells bearing the CD33 antigen will still allow regrowth of normal marrow cells of all lineages, presumably because of the presence of more immature antigen negative progenitors (25). Sabbath et al. (15) show that the CD33 antigen is expressed on leukemic colony-forming cells whereas other more mature markers are less commonly expressed. Finally, studies with ANLL marrow suggest it may be possible to purge leukemia cells from the bone marrow of many patients with ANLL using complement fixing antibodies to CD33 without destroying the ultimate normal progenitors (24). Several other antibodies with a less restricted distribution have also been described (14, 28, 29). In this and in an accompanying paper (30) we describe a new mouse monoclonal antibody, M195, which defines an antigen restricted to early myeloid cells, monocytic cells, and ANLL. The antigen appears to be carried on the CD33 protein. The antigen is not detectable on any other adult tissues and thus may be useful in the study of myelomonocytic differentiation and in the diagnosis and therapy of ANLL. This paper describes the distribution of the antigen on cell lines, normal tissues, and mature hematopoietic cells. The antibody's biological activity, affinity, and quantitative distribution on individual cells are presented. In the accompanying paper (30) we describe the distribution of the antigen on fresh leukemias and bone marrow progenitors and discuss its use as a diagnostic marker and its relationship to the CD33 antigen system.

MATERIALS AND METHODS

mAbs. mAb M195 was produced from hybridomas resulting from a fusion of SP2/0-Ag14 mouse myeloma cells and the spleen cells of a 5-week-old BALB/c mouse immunized with leukemia cells from a

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LEUKEMIA

patient with ANLL (FAB-M2). Supernatant fluids from cloned hybridoma cultures were screened against a panel of leukemia cell lines and the original ANLL leukemia cells using *Staphylococcus aureus* protein A (PA) erythrocyte rosetting (see below). The repeatedly subcloned M195 hybridoma was expanded in the peritoneal cavity of doubly pristane-primed (C57BL/6 × BALB/c) F1 mice.

M195 was purified on PA-Sepharose (Pharmacia) by affinity chromatography using sequential pH step elutions. Purity was determined on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie brilliant blue.

Control antibodies included mAb AJ2, reactive with a broadly expressed cell surface antigen (VLA), produced at Sloan-Kettering (31), and mAb M31 (reactive with the Lewis X antigen) developed in this laboratory (unpublished).

Screening of Hybridoma Supernatants. Four thousand cells (HL60) or the original immunizing ANLL cells) in 10 μl of RPMI with 10% fetal calf serum (FCS) were allowed to settle and attach to concanavalin A-coated (Pharmacia) Terasaki 60-well plates (NUNC) for 45 min at 20°C as described (32, 33). Hybridoma supernatants were tested for reactivity on these cells using PA-coated human O^- red blood cell rosettes as indicators (32).

Cells and Cell Lines. Heparinized peripheral blood samples and bone marrow aspirates were obtained from healthy volunteers and patients on the Leukemia Service at Memorial Hospital after informed consent. Mononuclear cells were separated on Ficoll-Paque (Pharmacia), and adherent cells were isolated from the nonadherent mononuclear cells by plastic adherence for 2 hr at 37°C. Polymorphonuclear leukocytes were purified from contaminating red blood cells after dextran sedimentation at 1 × *g* for 60 min by ammonium chloride lysis in Tris buffer at pH 7.2. Platelets were separated from the Ficoll-Paque interface cells by differential centrifugation. E-rosette-positive and negative fractions of mononuclear cells were separated after incubation with neuraminidase- (Calbiochem) treated sheep red blood cells (GIBCO), followed by Ficoll-Paque gradient centrifugation and lysis of red cells with ammonium chloride.

Hematopoietic cell lines (Table 1) and nonhematopoietic cell lines (Table 3) were obtained from the human tumor banks of the Human Cancer Immunology Laboratory at Sloan-Kettering Institute. 1F10 and 1F10 (mono), an HL60 subclone and its monocytic differentiated form, were the gift of Dr. Y. Cayre, Sloan-Kettering Institute (34).

Serologic Assays: Immune Rosetting. Antibody specificity was determined on adherent cell lines plated in 60 well Terasaki plates using Staphylococcus aureus PA or rabbit antimouse Ig-coated human O red blood cells prepared as described (32) as indicators. Suspension target cells were assayed using the same indicator cells except that the target cells were attached to Terasaki plates immediately before testing using Concanavalin A (33). This assay is sensitive to concentrations of mAb M195 of about 1 ng/ml binding to HL60. Cells were considered negative if no rosettes formed below an ascites dilution of 200 and absorption analyses were also negative. Ascites fluids were considered "weakly positive" on a cell line if greater than 50% of cells formed rosettes at dilutions between 200 and 100,000. "Weakly positive" cells were confirmed as reactive by absorption analysis (see below). Ascites from mice bearing hybridomas were considered positive with a cell line if rosetting of cells occurred at a dilution of greater than 100,000. If purified antibody was used, cells lines were scored "positive" for rosetting at concentrations below 50 ng/ml and "weakly positive" at concentrations of 50–500 ng/ml. Reactivity was also confirmed by direct radioimmunoassay and by complement fixation assays (see below).

Absorption Analysis. Two to ten million cells were washed in PBS and pelleted at 500 × *g* in a 5 × 50 mm glass tube and allowed to react with an equal volume of ascites diluted to a concentration four times that needed to form 50% rosettes on positive cells: HL60 cells or the immunizing ANLL cells. (This was typically a dilution of ascites of 100,000–200,000.) The absorption proceeded for 30 min at 4°C, the mixture was again pelleted at 500 × *g*. The supernatant was reacted with target cell lines in rosetting assays as described above.

Antibody-dependent Cellular Cytotoxicity (ADCC). Assays to determine if M195 was capable of mediating ADCC were conducted essentially as described by Welt et al. (35). Target cells were incubated in ^{51}Cr for 90 min and then washed of free ^{51}Cr. M195 antibody was added at concentrations of 1–100 μg/ml on ice, and fresh peripheral blood mononuclear cells were added at effector to target ratios of 10–40/1. Cells were incubated at 37°C for 6–18 hr and harvested using a Skatron cell harvester, and released ^{51}Cr was counted in a Packard gamma counter. Detergent lysed cells were used as a 100% control, and isotype matched irrelevant antibody treated cells were used as a negative control.

Radioiodination and Radioimmunoassays. Purified antibodies were labeled with Na-^{125}I (New England Nuclear) using chloramine-T to start and sodium metabisulfite to stop the reaction. Specific activity was between 2 and 10 μCi/μg of protein. Immunoreactivity was between 40 and 60% as determined by serial binding to an excess of live HL60 cells. Radioimmunoassays were conducted on 5 × 10^5 live cells in 100 μl RPMI with 10% FCS and preincubated 15 min with 2% heat inactivated normal rabbit serum to block nonspecific binding. Binding proceeded at 4°C for 90 min followed by three washes with RPMI/FCS. Bound radioactivity was measured in the cell pellets in a Packard gamma counter.

Preparation of F(ab)'2 Fragments. One mg of purified immunoglobulin was reacted at 37°C for 6 hr with immobilized pepsin beads (Pierce Chemicals) in acetate buffer at pH 4.5. The reaction was stopped by adjusting the pH to 8.8 and sedimenting the pepsin beads at 15,000 × *g* for 1 min. Undigested immunoglobulins and Fc fragments were removed by reaction with Protein A Sepharose (Pharmacia). Purity of fragments was determined by SDS-polyacrylamide gel fractionation followed by Coomassie blue staining.

Competition Radioimmunoassay for Blocking Antigen in Serum. Serum from patients with hematopoietic neoplasms was obtained from fresh clotted blood and stored at −70°C until use. The presence of blocking M195 antigen in sera was assayed by incubating 50 μl of freshly thawed serum with a dilution of ^{125}T-labeled mAb M195 IgG for 20 min at 4°C. M195 IgG was at a concentration sufficient for 50% maximal binding to 5 × 10^5 HL60 target cells. The cells were then added and the incubation continued for 60 min at 4°C followed by two washes with RPMI medium. Inhibition of M195 IgG binding was scored as the percent decrease in binding to HL60 as compared to mAb M195 incubated in the presence of 2% bovine serum albumin (BSA) in PBS and no competing sera.

Complement-mediated Cytotoxicity. Twenty-five μl of target cells at 2 × 10^6 cells/ml were mixed with 25 μl of complement and 25 μl monoclonal antibody at 4°C. The mixture was then incubated at 37°C with occasional shaking for 45 min. Live and dead cells were enumerated using trypan blue exclusion as an indicator.

Guinea pig serum and baby rabbit serum were purchased from PelFreeze; human sera were obtained from volunteers. All complement sources were stored at −70°C until use and not reused. Complement was used at the maximum concentrations not showing nonspecific lysis of the target cells: generally at 1:6–8 final dilution.

Indirect Immunoperoxidase and Immunofluorescence Assays. Histologically normal adult human tissues were obtained from surgical pathology specimens within 1–2 hr of resection. Several normal specimens of organs from several cases were used. Tissues were embedded in OCT compound after freezing in isopenthane/liquid N_2. Tissues were cut 4–8 μm thick, fixed in acetone, quenched with 0.1% H_2O_2, and blocked with either goat or horse sera. MAb M195 was used as supernatant, ascites, or purified IgG at 20 μg/ml. Positive and negative Ig controls were included in all studies. Goat anti-mouse IgG peroxidase conjugates (1:50 dilution) (Tago, Burlingame, CA) or biotinylated horse anti-mouse IgG with Avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, CA) were used as secondary reagents. Diaminobenzidine was used as a chromogen. For fluorescence studies, goat anti-mouse Ig fluorescein isothiocyanate conjugates (Becton-Dickenson) were used as secondary reagents.

Modulation of Cell Surface Antigen. Modulation of the cell surface

igen detected by mAb M195 after antibody binding was monitored complement mediated cytotoxicity (36). HL60 cells were incubated h various concentrations of M195 IgG for up to 3 hr at 37°C. ditional antibody and rabbit complement were added at several time nts and the amount of cell lysis was determined 45 min later.

)i[illegible]iation of HL60. A cloned variant of HL60, IF10 (34), and di[illegible]tiated monocytic variant (incubation with vitamin D_3 and rbal myristate acetate for 3 days to promote monocytic maturation) e used (34). Both cell lines were kindly provided by Dr. Y. Cayre.

RESULTS

)istribution of M195 Antigen on Hematopoietic Cell es. mAb M195 was selected for detailed study from a group several hundred hybridoma-produced antibodies generated n the fusion of a spleen from a mouse immunized with fresh : ANLL cells (FAB classification, M2). The antibody showed cific high titer binding in PA-rosette assays to the myeloid monocytic cell lines, HL60, KG1, IF10, U937, and the nocytic variant of IF10 (Table 1). mAb M195 was weakly tive with the erythroleukemic line K562 and not reactive n KG1a, an undifferentiated myeloid line. mAb M195 did react with 18 lines of B cell origin at various stages of erentiation nor with 10 lines of T cell derivation. One null phocytic line, N-ALL-1, was weakly reactive. Activated ells and activated T cells did not express antigen. Non- tive cell lines were confirmed as negative by absorption ys which can detect about 1 ng of M195 binding in 1,000,000 s; rosetting assays detect binding at antibody concentrations bout 1 ng/ml. mAb AJ2 was used as a positive control in e assays where most cells were M195 antigen negative. s panel of cell lines showed that among hematopoietic cells)5 was restricted to the nonlymphoid lineages: it was most ly [illegible]pressed on committed myeloid and monocytic cell s [illegible]ore weakly expressed on erythroid and earliest mye- cells. [illegible]able 3) were obtained from [illegible]

Quantitative Analysis of Binding to Myeloia [illegible]l Lines. In order to confirm the results of rosetting assays and absorption assays and to look at quantitative differences in the expression of the M195 antigen among the myeloid and monocytic cells, a sensitive radioimmunoassay using direct binding of ^{125}I-labeled purified M195 was used. Many of the hematopoietic cells have Fc receptors in addition to or instead of target antigen on their surfaces, and binding of radiolabeled IgG to these Fc receptors may confound the quantitative results of the radioimmunoassay. Therefore an F(ab)'2 fragment of M195 was prepared and used in the assays to confirm the number of antigenic sites.

Binding of M195 IgG to HL60 showed saturation and specificity (Fig. 1a). Scatchard analysis showed an avidity of binding of 3×10^9 liters/mol (Fig. 1b) for the IgG. The number of binding sites calculated from this curve was approximately 10,000 per live HL60 cell. Scatchard analysis of several lots of M195 IgG on different passages of HL60 gave equivalent results. Analysis of purified F(ab)'2 of M195 (Fig. 1c) showed similar avidity (10^9 liters/mol) cells and numbers of binding sites (10,000/HL60 cell), suggesting that binding activity was not significantly altered by protease digestion of the fragment.

Both the intact IgG and the F(ab)'2 fragment were used for radioimmunoassays on hematopoietic cell lines (Fig. 2). Nonspecific binding (binding of ^{125}I-M195 in the presence of excess unlabeled M195) under the conditions of this assay was approximately 200 pg (1600 molecules) per 5×10^5 cells. Therefore, only binding above this level was considered significant. Since the assay was done under saturating concentrations of M195 IgG or F(ab)'2, the total binding could be used to calculate the number of sites per cell. HL60, 1F10, and U937 had 6000–12,000 sites per cell. KG1 had about 3000 sites per cell. Binding to KG1a and K562 was not above the background of nonspecific binding (1600 sites), and the nonmyeloid cell lines were negative. The assays confirmed the specificity of M195

Table 1. Reactivity of M195 with Hematopoietic Cell Lines[a]

Cell	M195	AJ2 (positive control)
Myeloid		
K562, HL60	◑●	●
KG-1, KG1a,	●○	●●
1F10	●	●
Monocytic		
U937, 1F10 (mono)	●●	●●
THP-1	○	
Pre-B cells		
NALL-1, NALM-1	○○	●
NALM-6, NALM-16	○○	
B cells		
SK Ly-16,-18, Daudi,	○○○	●
ARA-10, SK DHL-2,Raji	○○○	●●
CCRF-SB, LICR/My-2	○○	●
BALL-1	○	
Myelomas		
Oda, U266, RPMI 8266	○○○	●
RCS, HAS, Brown	○○○	
EBV-transformed B cells (n = 15)	○	
T cells		
T-45, CCRF-CEM, Molt-4	○○○	●
TALL-1, MT-1, HUT-102	○○○	
RPMI 8402, CCRF-HSB2	○○	
p12/Ichikawa, HPB-ALL	○○	●
PHA blasts (n = 5)	○	

[illegible]e; ◑ = weakly positive; ○ = negative.
[a] [illegible]ined by direct Protein A and mixed heme-adherence rosetting and absorption assays as described in the text.

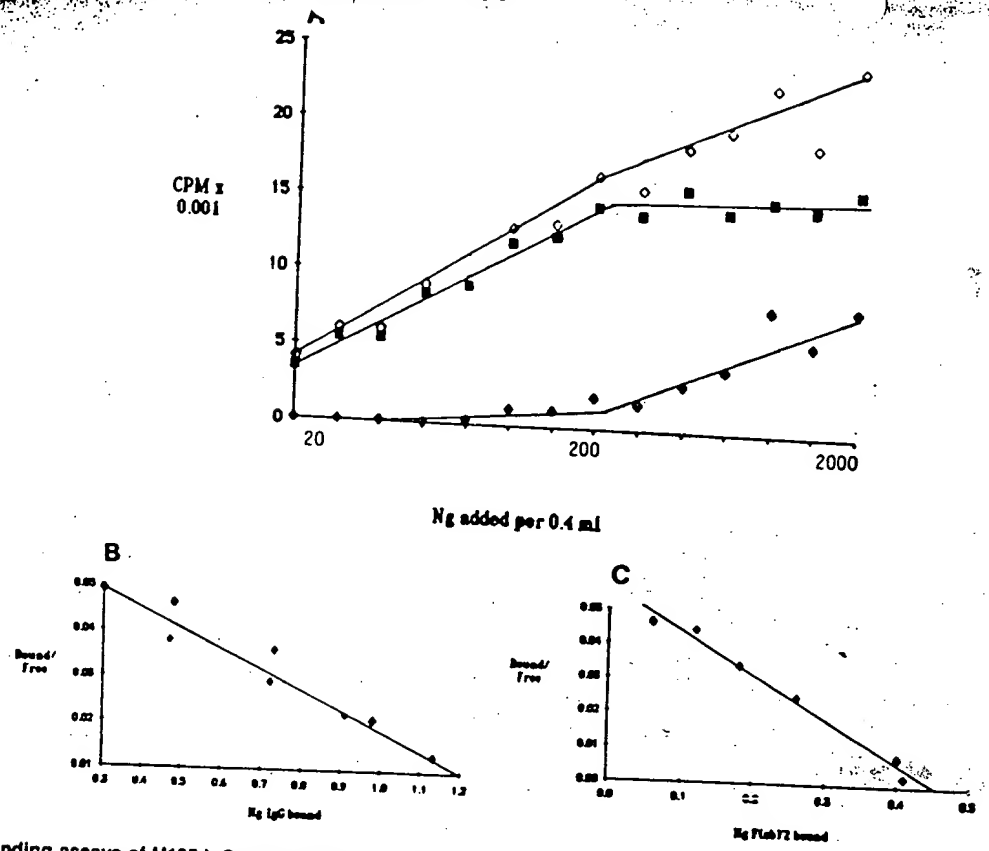

Figure 1. Radiobinding assays of M195 IgG and F(Ab)'2: Saturation and Scatchard analysis. Assays were conducted as described in Materials and Methods. A, Total (◇), nonspecific (binding of ^{125}I-IgG in the presence of excess unlabeled IgG (◆), and specific (■) binding of M195 IgG to HL60 leukemia cells. B, Scatchard plot of M195 IgG binding. C, Scatchard plot of M195 F(Ab)'2 binding.

for these myeloid and monocytic cells and showed that binding not Fc receptor related. The three cell lines positive by ting and absorption had similar quantities of M195 antigen expression.

Reactivity with Fresh Normal Hematopoietic Cells. M195 was tested by absorption analysis for reactivity with live peripheral blood elements and cells derived from the major hematopoietic organs (Table 2). mAb M31 was used as a positive control. No reactivity was seen with M195 on any of these cell types.

Quantitative Analysis of Binding to Hematopoietic Cells. Direct radioimmunoassays were performed on fresh he-

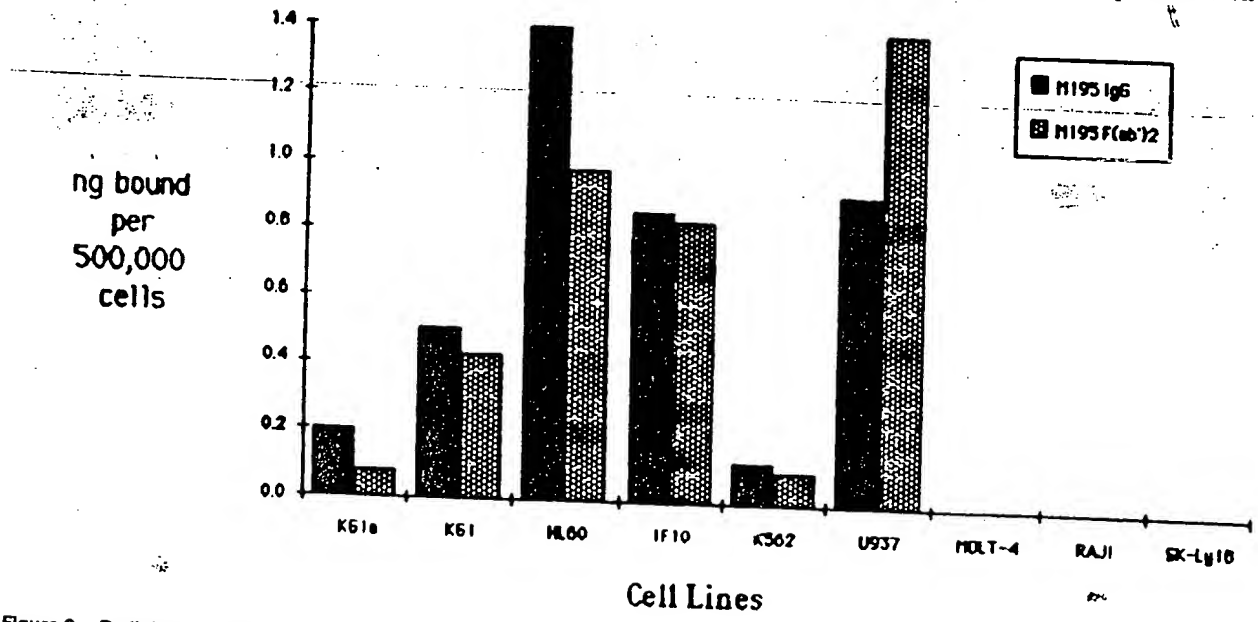

Figure 2. Radioimmunoassay of M195 IgG and F(Ab)'2 on cell lines of hematopoietic origin. ^{125}I-M195 binding was determined at saturation as described in Materials and Methods. Nonspecific binding was 0.2 ng/500,000 cells. Only specific binding is shown: IgG, ■; F(Ab)'2, ▨.

Table 2. Reactivity of M195 with Fresh Hematopoietic Cells[a]

Cell Type	Assay Type		
	Absorption	Radioimmunoassay	Complement Lysis
T-enriched PBL[b]	–	–	–
B-enriched PBL	–	–	–
Granulocytes	–	–	–
Adherent monocytes	–	+	+
Platelets	–	–	ND[c]
Red blood cells	–	–	ND
Nonadherent PBMC	ND	–	–
Splenic T enriched	ND	–	–
Splenic B enriched	ND	–	–
Splenic mononuclear	–	ND	–[d]
Bone marrow mononuclear	–	–	–[d]
Lymph node mononuclear	–	ND	ND
Fetal thymocytes	–	ND	ND

[a]Conducted as described in the text.
[b]PBL = peripheral blood lymphocytes; PBMC = peripheral blood mononuclear cells.
[c]ND = not done.
[d]Nonadherent cells.

matopoietic cells to confirm reactivity and quantitate binding (Table 2). Red blood cells, platelets, spleen cells, bone marrow cells, and peripheral blood mononuclear cells were negative. Polymorphonuclear leukocytes showed binding to the intact IgG at about 800 sites per cell above background but did not show significant binding to the F(Ab)'2 fragment suggesting that even this minimal binding was via the Fc receptors. Peripheral blood adherent cells (macrophages) were positive and binding to the F(Ab)'2 showed about 5000 antigen sites per cell. Binding to peripheral blood E-rosette negative cells was marginally above background, possibly due to the presence of a small percentage of macrophages contained in this population. With the exception of macrophages, the direct radioimmunoassays shown here confirmed the specificity analysis by absorption. The lack of reactivity with macrophages in the absorption assay may be due to the inability to obtain the large quantity of viable cells containing enough antigen necessary to absorb M195 (1,000,000 macrophages with 5000 antigen sites per cell would absorb only about 1 ng of antibody). Lack of binding in these radioimmunoassays would not rule out the presence of some M195 positive cells within a large heterogeneous population as in bone marrow, for example.

Complement-mediated Cytotoxicity Assays. Complement-mediated cytotoxicity was also used to confirm specificity. Assays were first done to determine if mAb M195 was capable of killing cells in the presence of rabbit, guinea pig, and human sera as sources of complement. Enzyme-linked immunosorbent assays showed mAb M195 to be an IgG2a class immunoglobulin, which is generally able to fix complement. Using HL60 as targets, M195 was capable of killing cells in the presence of guinea pig and rabbit complement but not human complement. In the presence of human complement, killing rarely occurred and was only 10–15% above background at its highest. Cell lines not expressing the antigen were not killed. No killing occurred in the absence of antibody or a source of complement.

Cytotoxicity was antibody concentration dependent and complement concentration dependent (Fig. 3). However, at concentrations of 10 μg/ml or greater of M195, nearly all cells were killed even with rabbit serum diluted 30-fold.

The complement assay was used to confirm the specificity analysis derived from the absorption assays and radioimmunoassays (Table 2). Complement assays are not confounded by Fc receptor binding and are able to determine percentages of cells within a large population which are antigen positive. Assays were conducted at 10 and 100 μg/ml M195 with rabbit serum diluted to 1:18 final concentration. HL60 and fresh monocytic leukemias were used as positive controls and B cell lineage RAJI cells and chronic lymphocytic leukemia cells were used as negative controls. Complement and antibody alone controls were also included. Background killing was between 1–5% in the controls without antibody or complement and 5–10% in the spleen E-rosette negative cells. Because of this background of several percent, it is not possible to determine if positive cells are present in a sample at this level or lower.

Only one population of mature normal hematopoietic cells showed killing above background using M195 and rabbit complement: peripheral blood adherent cells. Among three samples of adherent cells, 35–50% of cells were killed, showing that a subpopulation of these cells expressed the M195 antigen. This assay confirmed the radioimmunoassay data.

In chronic myelogenous leukemia (CML) mononuclear cells, a low percent of cells (5–6%) were killed above background (not shown). The cells comprising the CML mononuclear cell population include blasts through band forms with a predominance of the more mature myeloid cells. Morphologic analysis of these cells before and after antibody and complement treatment did not show which cells, if any, had been selectively

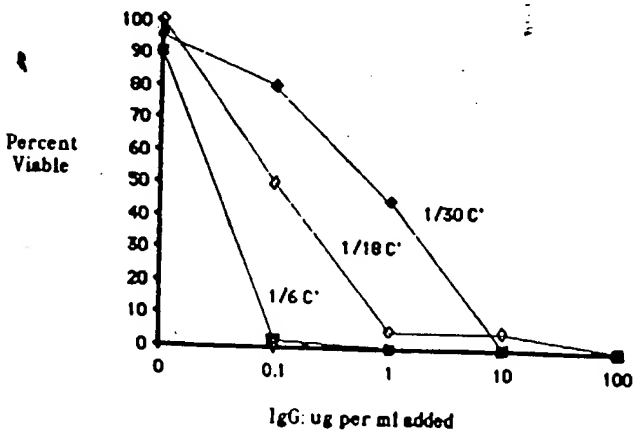

Figure 3. Complement cytotoxicity by M195 IgG on HL60 cells using rabbit complement. The concentration of rabbit complement is shown in the figure: final dilution of 1/6 (■), 1/18 (◇), and 1/30 (◆). The assay was conducted as described in Materials and Methods.

cells. Because peripheral blood cells from patients with CML represent the full spectrum of maturing myelogenous cells, this lack of significant cytotoxicity confirms the lack of reactivity of M195 with the vast majority of adult myelogenous cells.

Reactivity of M195 with Differentiated HL60 Cells. 1F10 cells and differentiated monocytic 1F10 cells were provided by Dr. Yvon Cayre. One hundred percent of the 1F10 cells became morphologically changed and adherent. The reactivity of M195 was tested by both rosetting and radioimmunoassays before and after differentiation. In the differentiated monocytic 1F10 cells there was a 40% loss of antigen expression by radioimmunoassay. Rosetting assays remained positive but the titer of binding dropped 10-fold. Quantitative binding to the differentiated 1F10 was similar to fresh normal adherent monocytes, suggesting that the loss of antigen with monocytic differentiation among fresh hematopoietic cells was paralleled by this model line in vitro.

ADCC Assays. M195 did not show any ability to mediate ADCC against HL60 cells or U937 under the conditions described in Materials and Methods. These cells are the highest expressors of the antigen among those tested.

Reactivity of M195 with Nonhematopoietic Cell Lines. M195 was tested for reactivity with 70 cell lines derived from a wide spectrum of cancers (Table 3). No reactivity was seen. Monoclonal antibody AJ2 was included as a positive control and was positive in every case tested. Therefore, the M195 antigen appears to be restricted to hematopoietic cells.

Tissue Distribution of M195. The reactivity of M195 with human tissues was determined in indirect immunofluorescence and indirect immunoperoxidase assays on fresh frozen tissue (Table 4). Among 25 different tissue types, reactivity was seen only with trophoblast. This reactivity was predominantly cytoplasmic (Fig. 4). These data on fresh tissue are consistent with the specificity data obtained from the assays with cell lines above.

M195 Reactivity with Fresh Leukemias. M195 reacted with most myelogenous leukemias and rarely with lymphoid leukemias in rosetting assays. Because of the nature of the rosetting assay, it was not possible to determine which cells were reactive or what percentage of blasts were positive. These issues and a detailed analysis of the specificity and activity of M195 in comparison to standard cell surface markers are presented in the accompanying paper (30).

M195 Blocking Antigen in Serum. In order to determine if the M195 antigen was shed into sera from hematopoietic cells, sera from people with a variety of leukemias and lymphomas or from healthy individuals were tested for soluble antigen capable of blocking of the binding of radiolabeled mAb M195 to HL60 cells (Table 5). Three of 39 human sera blocked binding significantly. The blocking was not complete. One serum was from a patient with CML. Two sera of six patients with acute lymphocytic leukemia partially blocked binding. The leukemia cells from both of these patients were not reactive with M195 antibody suggesting that the blocking antigen was not shed from these cells or that the blocking activity was not specific. These data suggest that M195 antigen in the serum would not be capable of preventing mAb M195 from reaching target cells. Because the sensitivity of this assay is about 200 ng/ml of M195, it is possible that M195 is expressed at lower levels than this in sera. In addition, monovalent antigen with low avidity for the M195 IgG may be present but unable to block binding.

Antigenic Modulation. The ability of M195 to induce modulation of the antigen from the surface of HL60 cells was studied using complement-mediated cytotoxicity. HL60 cells were reacted with M195 at various concentrations, and the ability of M195 to kill the cells with added rabbit complement was measured versus time (Fig. 5). At the highest antibody concentrations complete modulation occurred within 3 hr. That is, the addition of complement to cells preincubated with mAb

Table 3. Reactivity of M195 with Non-Hematopoietic Cell Lines[a]

		M195	AJ2 (positive control)
Astrocytomas	SK-MG-1,-2,-3, -4,-6,-7,-9	○○○	●
	-12,-15,17,-23	○○○	● ●
Bladder cancers	T-24, 253J,5637	○○○○	● ●
Breast cancers	SK-BR-3,-5,-7	○○○	
	BT-20,MCF-7	○○○	
Cervical cancers	CC-A, CC-B, HT-3	○○	◒
	C41	○○○	● ● ●
Choriocarcinomas	GCC-SV(c), Lu-75(c)	○	●
Colon cancers	SW-403,-480,-620	○○	
	-116 -1417	○○○	● ● ●
	HT-29,SK-CO-10	○○	● ●
	CaCo-2,HCT-15	○○	● ●
Lung cancers	SK-LC-1,-4,-6	○○	● ●
	-8,9,-10,-17	○○○	● ●
	Calu-1,-6,Sk-Lu-1	○○○○	● ●
	SK MES-1	○○○	● ● ●
Melanomas	SK MEL-13,-23,-28	○	●
	-29,-37,-93	○○○	● ● ●
	-173,MeWo	○○○	● ● ●
Neuroblastomas	SK-N-MC,PNDW	○○	●
Ovarian cancers	SK-OV-3,OV-2774	○○	● ●
Pancreatic	ASPC-1,-2	○○	●
Renal cancers	SK-RC-1,-2,-7	○○	● ●
	-8,-9,-20,-28,	○○○	●
	-29,-45,-48	○○○○	● ●
Uterine cancer	ME 180, SK UT-1	○○○	● ● ●
		○○	● ●

[a] Conducted as described in Table 1.

Table 4. Tissue Distribution of M195[a]

Tissue	Fluorescence	Peroxidase
Adrenal	○	○
Bladder	○	○
Blood vessels	○	○
B[illegible]	○	○
B[illegible]	○	○
Capillaries	○	○
Cervix	○	○
Colon	○	○
Heart	○	○
Kidney	○	○
Liver	○	○
Lung	○	○
Lymph node	○	○
Ovary	○	○
Pancreas		○
Placenta	○	○
Prostate	○	○
Skin	○	○
Small intestine	○	○
Stomach	○	○
Testis	○	○
Thyroid	○	○
Trophoblast		●
Ureter	○	○
Uterus	○	○
HL60 (positive control)	●	●

○ = negative; ● = positive staining.
[a]Conducted as described in the Materials and Methods.

Table 5. M195 Blocking Factors in Sera of Patients with Leukemia

Serum Source	Number Tested	Number Blocking[a]
Normal	6	0
AML	13	0
CML	6	1 (52%)[b]
ALL	6	2 (56%, 67%)
NHL/CLL	8	0
Rabbit, mouse, horse	5	0

[a]A serum able to reduce by 50% or more, direct binding of 200 ng/ml radioiodinated M195 to HL60 target cells.
[b]The percent reduction by each positive serum.

M195 for 3 hr resulted in no killing. Modulation was incomplete in cells exposed to lower mAb IgG concentrations. Other studies (to be published elsewhere) demonstrated that the modulation occurred via antigen internalization after antibody binding.

Biochemical Nature of the M195 Antigen. Treatment of HL60 cells with 100°C for 1 min eliminated all binding activity in radioimmunoassays and rosetting assays. This suggested that the antigen epitope is carried on a protein. However, treatment with trypsin, protease, and neuraminidase had no effects on binding of mAb M195 to HL60 cells. These experiments, therefore, did not confirm the biochemical nature of the antigen. Repeated attempts to immunoprecipitate the antigen from ^{35}S-methionine-labeled cells or cells surface-labeled with iodine-125 using lactoperoxidase were unsuccessful. Western immunoblotting on HL60 extracts were also negative. Although we were unable to identify the target, other data shown in the accompanying paper (30) indicated that the antigen was carried on the CD33 protein.

DISCUSSION

This paper details the specificity of a new mouse mAb, M195, which is reactive with myelogenous leukemias, early myeloid cells and some monocytic cells. Qualitative and quantitative analyses of the mAb's binding, its biological activity, and its immunological functions are described.

[illegible]. Indirect immunoperoxidase assay of M195 on trophoblast cells. A, photomicrograph of M195 IgG binding to trophoblast. B, Control IgG binding to trophoblast cells.

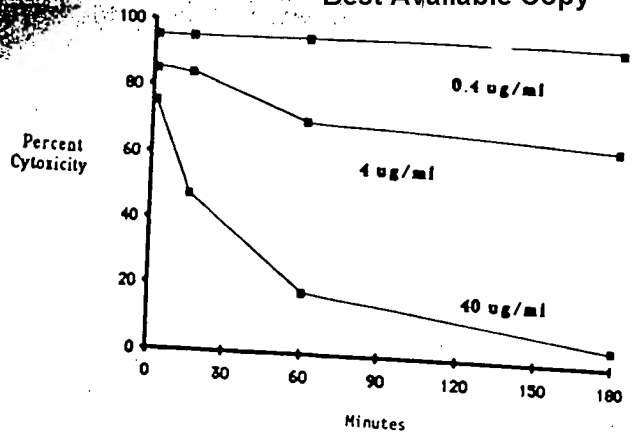

Figure 5. Antigenic modulation after exposure of HL60 cells to mAb M195. The assay was conducted as described in Materials and Methods. M195 IgG was added at the concentrations shown in the figure and allowed to incubate at 37°C for the times indicated on the x axis. Cells were then tested for lysis by an additional aliquot of M195 IgG with rabbit complement at 37°C for 45 min. Cytotoxicity after this second addition is shown on the y axis.

Since two potential uses of M195 are diagnosis and in vivo therapy of ANLL, a comprehensive definition of its reactivity with all tissues and cells of the body was undertaken. Several assays were used in the specificity analysis of M195 on fresh cells and cell lines. Rosetting assays which are sensitive enough to detect 1 ng of mAb M195 per ml were used initially for specificity analysis. Direct radioimmunoassays using iodine-125 labeled IgG and F(Ab)'2 were used next in order to quantitate the number of antigen sites expressed on various positive cells. The F(Ab)'2 has the advantage of defining non-Fc receptor binding quantitatively. Finally, a complement fixation assay was used to analyze reactivity. Since biological activity after binding to antigen in an appropriate fashion is required in this assay, the effects of nonspecific binding are reduced. Indirect immunofluorescence followed by confirmation with indirect immunoperoxidase assays were used to define M195 antigen expression on a broad spectrum of normal tissues. These results supported data obtained from the rosetting and absorption analysis on cell lines. Because the tissues were frozen sectioned and fixed, binding to cytoplasmic as well as membrane antigen could be detected in these assays.

M195 was found to bind specifically only to myeloid cell lines and monocytic cells. Lymphoid cells, including peripheral blood T and B cells, lymph node, spleen, and bone marrow cells, T and B cell lines representing pre-B, early B, B, and late B cell stages and T cell leukemias, and activated fresh B and T cells, did not express the M195 antigen. Red cells and platelets were also negative. Among 95 nonhematopoietic cell lines and nonhematopoietic tissues, only trophoblasts were reactive with M195. This activity appeared to be cytoplasmic. The presence of myeloid antigens in the cytoplasm of choriocarcinoma cells but not normal trophoblast has been reported (37), but its significance is unknown.

Within the myelomonocytic lineage, the distribution of M195 antigen was even further restricted. Polymorphonuclear leukocytes were not reactive nor could significant binding be demonstrated in normal bone marrow mononuclear cells. A small percentage of cells from the peripheral blood mononuclear cells from patients with chronic myelogenous leukemia were positive. These samples contain largely granulocytic precursors up to the band stage. The lack of reactivity with polymorphonuclear leukocytes and this slight reactivity with CML suggests that the vast majority of mature and precursor myeloid cells do not express M195 antigen. In contrast, myeloid leukemia lines and fresh myeloid leukemias were strongly positive. Cell lines representing the earliest myeloid cells or erythroid cells were either negative or less positive than the myeloid cell lines representing later leukemias. These data place the M195 antigen expression to cells in the early to middle part of myeloid differentiation: the antigen is not present at first and is lost as the cells mature toward granulocytes.

Among monocytic cells, M195 reacted with both monocytic leukemia lines and a fraction of mature peripheral blood adherent cells. It was present on the HL60 variant, IF10, and in reduced amounts after monocytic differentiation of IF10 with vitamin D3 and phorbal esters. Likewise, AMOL blasts contained about 10,000 sites (30) compared to macrophages with 5000 sites. Therefore, like its expression on granulocytic precursors, the expression of the M195 antigen or monocytic cells appears to be maturation dependent.

Analysis of quantitative binding to HL60 cells gave an avidity of binding of the M195 IgG of 3×10^9 liters/mol. Binding was saturable and cell number dependent. These data showed that positive cell lines expressed about 10,000 antigen sites per cell. Therefore, M195 was rather weakly expressed compared to many other cell surface antigens. Although we have been unable to identify the target antigen of mAb M195, several of its features suggest it is a polypeptide. The antigen is heat labile; there are small numbers expressed on the surface. The antigen is rapidly modulated after antibody binding, and the antigen was detected by an IgG2a (which in this laboratory rarely identify carbohydrate).

The extremely restricted expression of this antigen among the cell types tested, the biochemical features noted above, including rapid modulation and internalization, and the small number of sites per cell all suggested that the M195 target may be a receptor important in growth and differentiation of myeloid progenitors. However, studies of the effects of M195 alone on the growth of myeloid cell lines, peripheral blood mononuclear cells (data not shown), and colony forming units (30) have not so far shown any stimulating or inhibiting effects of the mAb.

mAbs reactive with restricted myeloid antigens may be useful in at least four areas.

A) *Study of the Growth and Differentiation of Myeloid and Monocytic Cells*. Of the many antigen and antibody systems that have been described in myelomonocytic differentiation, three systems which have defined different states of myeloid maturation have been most widely studied: the CD34 system (mAbs MY10, 12.8, 3C5) (19–21) which identifies a gp115 found on the earliest hematopoietic progenitors, both lymphoid and myeloid, and which rapidly disappears upon differentiation is also found on some nonhematopoietic tissues including endothelium. mAbs to this antigen have been used to purify progenitors for reconstitution of bone marrow (18). The CD33 antigen system (mAbs MY9, L4F3, L1B2) (24–27) identifies a gp67 (17) restricted to early myeloid and monocytic cells. It is absent from the earliest hematopoietic progenitors and other normal tissues and has been used to eliminate leukemia cells, while sparing the ultimate progenitors, from bone marrow. The CD15 antigen system (multiple mAbs) identifies the Lewis X antigen found on granulocyte colonies from the day 7 stage on and increases expression as cells mature to the polymorpho-

[redacted] cell. This antigen is also widely distributed throughout [redacted] tissues (38).

The distribution of the M195 antigen detailed in this paper ows it to fall into the myelomonocytic-restricted second cat- or[redacted] competition binding studies and binding to CD33 trans- [redacted] iscussed in the accompanying paper) (30) demonstrated at M195 was carried on the CD33 protein. However, cotyping fresh leukemias showed that the antigen detected by mAb 195 was not identical to the other CD33 antigens (30).

B) *Diagnosis of Hematopoietic Neoplasms.* mAbs useful in agnostic applications must be lineage specific, but not nec- arily stage specific. For this reason, the CD34 antigen which also present on lymphoid cells is less useful than the mye- nocytic antigen systems CD13 and CD15 or the monocytic cific antigens CD14 (27, 39, 40). M195 was restricted to elomonocytic cells and is being tested for use in the clinical gnosis of ANLL at Memorial Hospital (see accompanying er) (30).

C) *Purging of ANLL from Bone Marrow.* In order to be ful in bone marrow purging, in addition to being myelo- nocytic specific, the mAb must spare the ultimate progenitor l. Reactivity with other tissues outside of the bone marrow not important. The ability to fix complement is important new methods to kill cells with toxins (41) or remove them h magnetic beads (42) may reduce this requirement. CD15 bodies have proven most useful in this application and are linical trials currently (12). CD33 antibodies may be even e useful, if adequate recovery of the bone marrow progen- s can be assured. M195, which rapidly and efficiently kills emic cells with rabbit complement, might be successfully lied to this problem.

D) *Therapy with mAb in Vivo.* This application is most dif- lt [redacted] optimally requires limited reactivity with normal [redacted] addition to the criteria described above. Of the many gen systems described for myelomonocytic cells, CD33 ears most suited for this application in vivo. M195 may be l in this application, but its demonstrated lack of cytotox- in the presence of human complement or PBMC in vitro t require that the mAb carry a cytotoxic isotope or toxin e effective. Since the antigen and antibody are rapidly nalized, this therapeutic modality may be feasible and in- igations of this application are underway.

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REFERENCES

oon KA, Todd RF. Immunologic classification of leukemia and ymphoma. Blood 1986;68:1–31.

Alvey PL, Greaves MF. A computer program for interpreting mmunophenotypic data as an aid to the diagnosis of leukemia. eukemia 1987;1:527–540.

Vilson WEC, Walker IR, Saeed N, McBride JA. Classifying cute leukemia by immunophenotyping. A combined FAB- mmunologic classification of AML. Blood 1986;68:1355–1362.

riffin JD, Linch D, Sabbath F, Larcom P, Schlossman SF. A [redacted] nal antibody reactive with normal and leukemic human [redacted] progenitor cells. Leuk Res 1984;8:521–534.

5. Dinndorf PA, Andrews RG, Benjamin D, Ridgway D, Wolff L, Bernstein ID. Expression of normal myeloid-associated antigens by acute leukemia cells. Blood 1986;67:1048–1053.
6. Pessano S, Palumbo A, Ferrero D, Pagliardi GL, Bottero L, Lai SK, Meo P, Carter C, Hubbell H, Lange B, Rovera G. Subpopulation heterogeneity in human acute myeloid leukemia determined by monoclonal antibodies. Blood 1984;64:275–281.
7. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Bevendga RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557–563.
8. Van der Reijden HJ, Van Rhenen DJ, Lansdorp PM, Van't Veer MD, Langenhuijsen MMAC, Engelfriet CP, Von dem Borne AEGKr. A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 1983;61:443–448.
9. Linch DC, Allen C, Beverley PCL, Bynoe AG, Scott CS, Hogg N. Monoclonal antibodies differentiating between monocytic and nonmonocytic variants of AML. Blood 1984;63:566–573.
10. Drexler HG, Minowada J. The use of monoclonal antibodies for the identification and classification of acute myeloid leukemias. Leuk Res 1986;10:279–290.
11. Ball ED, Bernier GM, Cornwell GG, McIntyre OR, O'Donnel JF, Fanger MW. Monoclonal antibodies to myeloid differentiation antigens: *In vivo* studies of three patients with acute myelogenous leukemia. Blood 1983;62:1203–1210.
12. Ball ED, Mills LE, Coughlin CT, Beck R, Cornwell GG. Autologous bone marrow transplantation in acute myelogenous leukemia: *in vitro* treatment with myeloid cell-specific monoclonal antibodies. Blood 1986;68:1311–1315.
13. Griffin JD, Lowenberg B. Clonogenic cells in acute myeloblastic leukemia. Blood 1986;68:1185–1195.
14. Lange B, Ferrero D, Pessano S, Palumbo A, Faust J, Meo P, Rovero G. Surface phenotype of clonogenic cells in acute myeloid leukemia defined by monoclonal antibodies. Blood 1984;64:693–700.
15. Sabbath KD, Ball ED, Larcom P, Davis RB, Griffin JD. Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 1985;75:746–753.
16. Lowenberg B, Bauman JGJ. Further results in understanding the subpopulation structure of AML: clonogenic cells and their progeny identified by differentiation markers. Blood 1985;66:1225–1232.
17. Peiper SC, Lemons RS, Ashmun RA, Look AT. DNA-mediated transfer of the gene encoding p67 (CD33), a myeloid differentiation antigen recognized by the MY9, L1B2, and L4F3 monoclonal antibody. In: McMichael AJ, ed. Leucocyte typing III. New York: Oxford University Press, 1987:622–625.
18. Berenson RJ, Bensinger WI, Kalamasz D. Positive selection of viable cell populations using avidin-biotin immunoadsorption. J Immunol Methods 1986;91:11–19.
19. Katz FE, Tindle R, Sutherland DR, Greaves MF. Identification of a membrane glycoprotein associated with hematopoietic progenitor cells. Leuk Res 1985;91:191–198.
20. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 1984;133:157–165.
21. Strauss LC, Rowley SD, LaRussa VF, Sharkis SJ, Stuart RK, Civin CI. Antigenic analysis of hematopoiesis. V. Characterization of My-10 antigen expression by normal lymphohematopoietic progenitor cells. Exp Hematol 1986;14:878–886.
22. Askew DS, Eaves AC, Eaves CJ, Takei F. Restricted expression of a new acute myelogenous leukemia-associated antigen (NHL-30.5) on normal hemopoietic progenitor cells. Blood 1986;67:1098–1102.
23. Askew DS, Eaves AC, Takei F. NHL-30.5: a monoclonal antibody reactive with an acute myeloid leukemia (AML)-associated antigen. Leuk Res 1985;9:135–145.

24. Bernstein ID, Singer JW, Andrews RG, Keeting A, Powell JS, Bjornson BH, Cuttner J, Najfeld V, Reaman G, Raskind W, Sutton DMC, Fialkow PJ. Treatment of acute myeloid leukemia cells *in vitro* with monoclonal antibody recognizing a myeloid differentiation antigen allows normal progenitor cells to be expressed. J Clin Invest **1987**;79:1153–1159.
25. Andrews RG, Takahashi M, Segal GM, Powell JS, Bernstein ID, Singer JW. The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. Blood **1986**;68:1030–1035.
26. Andrews RG, Torok-Storb B, Bernstein ID. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood **1983**;62:124–132.
27. Griffin JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest **1981**;68:932–941.
28. Peng R, Al-Katib A, Knowles DM, Lu L, Broxmeyer H, Tolidjian B, Chiao J-W, Koziner B, Wang CY. Preparation and characterization of monoclonal antibodies recognizing two distinct differentiation antigens (Pro-Im1, Pro-Im2) on early hematopoietic cells. Blood **1984**;64:1169–1178.
29. Wisniewski D, Knowles R, Wachter M, Strife A, Clarkson B. Expression of two natural killer cell antigens, H-25 and H-366, by human immature myeloid cells and by erythroid and granulocytic/monocytic colony-forming units. Blood **1987**;69:419–429.
30. Scheinberg DA, Tanimoto M, McKenzie S, Strife A, Old LJ, Clarkson BD. Monoclonal antibody M195: a diagnostic marker for acute myelogenous leukemia. Leukemia **1989** (in press).
31. Cairncross JC, Mattes MJ, Beresford HR, Albino AP, Houghton AN, Lloyd KO, Old LJ. Cell surface antigens of human astrocytoma defined by mouse monoclonal antibodies: identification of astrocytoma subsets. Proc Natl Acad Sci USA **1982**;79:5641–5645.
32. Shiku H, Takahashi T, Oettgen HF, Old LJ. Cell surface antigens of human malignant. II. Serological typing with immune adherence assays and definition of two new surface antigens. J Exp Med **1976**;144:873–881.
33. Mattes JM, Tanimoto M, Pollack MS, Maurer DH. Preparing monolayers of non-adherent mammalian cells. J Immunol Methods **1983**;61:145–150.
34. Cayre Y, Raynal MC, Darzynkiewicz Z, Dorner MH. Model for intermediate steps in monocytic differentiation. Proc Natl Acad Sci USA **1987**;84:7619–7623.
35. Welt S, Carswell EA, Vogel C-W, Oettgen HF, Old LJ. Immune and non-immune effector function of IgG3 mouse monoclonal antibody R24 detecting the disialoganglioside GD3 on the surface of melanoma cells. Clin Immunol Immunopathol **1987**;45:214–229.
36. Old LJ, Stockert E, Boyse EA, Kim JH. Antigenic modulation: loss of TL antigen from cells exposed to TL antibody. Study of the phenomenon *in vitro*. J Exp Med **1968**;127:523–539.
37. Berkowitz RS, Umpierre SA, Goldstein DP, Andersen DJ. Cross reactivity of monoclonal antibodies directed against lymphocyte markers with trophoblast cells of normal placenta, hydatidiform mole, and gestational choriocarcinoma. Gynecol Oncol **1988**;29:94–100.
38. Sakamoto J, Furukawa K, Cordon-Cardo C, Yin BWT, Rettig WJ, Oettgen HF, Old LJ, Lloyd KO. Expression of Lewis (a), Lewis (b), X and Y blood group antigens in human colonic tumors and normal tissue and in human tumor-derived cell lines. Cancer Res **1986**;46:1553–1561.
39. Drexler HG, Sagawa K, Menon M, Minowada J. Reactivity patterns of myeloid monoclonal antibodies with emphasis on MCS-2. Leukemia Res **1986**;10:17–23.
40. McMichael AJ, ed. Leukocyte typing III. New York: Oxford University Press, **1987**:577–602.
41. Krolick KA, Villemez C, Isakson P, Uhr JW, Vitetta ES. Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain ricin. Proc Natl Acad Sci USA **1980**;77:5419–5423.
42. Shimazaki C, Wisniewski D, Scheinberg DA, Atpodien J, Strife A, Gulati S, Fried J, Wisniewolski R, Wang CY, Clarkson BD. Elimination of myeloma cells from bone marrow using monoclonal antibodies and magnetic immunobeads. Blood **1988**;72:1248–1254.

Monoclonal Antibody M195: A Diagnostic Marker for Acute Myelogenous Leukemia

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Monoclonal antibody (mAb) M195 is a mouse IgG2a reactive with a myelomonocytic differentiation antigen found on early myeloid cells and monocytes. The reactivity of M195 with fresh hematopoietic neoplasms in the blood or bone marrow from 227 patients at Memorial Hospital was determined by flow cytometry. M195 was positive on 67% of 61 myeloblastic leukemias. Seventy percent of Tdt-negative ANLL and 30% of Tdt-positive ANLL were positive; 100% of CMMOL and 100% of CML in myeloblastic crisis or accelerated phase were positive. In contrast, M195 was positive on only 8% of 51 lymphoblastic leukemias and 1% of 70 other nonmyeloid samples. M195 binding did not correlate well with FAB classification of ANLL. The pattern of reactivity of M195 was similar but not identical to that of MY9 (CD33) on the same cases (83% concordance). Cross-blocking of M195 binding by MY9 and L4F3 (CD33) was demonstrated. M195 may bind to a different epitope on the same protein antigen. The presence of both MY9 and M195 positivity on a leukemia sample had a 98% specificity of diagnosing ANLL, which was greater than MY9 alone (88%) or M195 alone (92%). Assays of granulocytic-monocytic and erythroid colony-forming units showed M195 to be present on these hematopoietic progenitors. This pattern of reactivity of M195, together with its lack of reactivity with mature granulocytic elements or with adult tissues, make it a candidate for therapy of ANLL in vivo.

INTRODUCTION

MONOCLONAL antibodies (mAb) reactive with differentiation antigens present on myeloid cells and their progenitors are being used to study hematopoietic differentiation, to identify acute nonlymphoid leukemia (ANLL), to study the effects of hematopoietic growth factors, to purge bone marrow of leukemia cells, and for therapy in vivo (1–15). In our accompanying paper we describe a new mouse monoclonal antibody, M195, which detects an antigen found on early myeloid cells, monocytes, and ANLL cells but not on cells of other hematopoietic or nonhematopoietic lineages (16). The antigen described has several features in common with the myelomonocytic antigen CD33 (4, 5) which is found on early myeloid cells and ANLL cells but not on the ultimate progenitor cells (17), a characteristic which may allow selective killing of ANLL cells (18). In this paper we describe the specific reactivity of M195 with ANLL among 227 different fresh hematopoietic neoplasms. The reactivity was similar but not identical to that of MY9 (CD33). Cross-blocking of the binding of these two antibodies to target cells was found. In combination with MY9, M195 showed 98% specificity in diagnosing ANLL by flow cytometry of clinical specimens. M195 bound to most CFU-GM, as measured by colony forming assays. This pattern of reactivity of M195, together with its lack of reactivity with adult tissues (16) make mAb M195 a candidate for therapeutic trials in vivo.

MATERIALS AND METHODS

Monoclonal Antibodies. M195, a mouse IgG2a, was prepared in this laboratory as described (16). The following mAbs were purchased from Coulter Immunology (Hialeah, FL): MY9, an IgG2b, (CD33); B4, an IgG1, (CD19); B1, an IgG2A, (CD20); I_2 or I_3, IgG2As (anti-HLA-DR); MY4, an IgG2b, (CD14); and MY7, an IgG2b, (CD13). These were either obtained as fluorescein isothiocyanate conjugates or pure immunoglobulins. The following mAb were purchased from Becton-Dickinson (Mountain View, CA): MY10, an IgG1, (CD34) and goat anti-mouse Ig fluorescein isothiocyanate conjugate of F(ab)'2. L4F3, IgM, (CD33) ascites was the gift of Dr. Irwin Bernstein. M31, IgM, (CD15) and OKB7, IgG2B, (CD21) from a hybridoma provided by Ortho Biotech (Raritan, NJ) were prepared in this laboratory.

Flow Cytometry. Five million fresh live mononuclear cells from bone marrow or blood from patients on the Hematology-Lymphoma Service at Memorial Hospital were incubated in 0.1 ml final volume with the fluorescein conjugated monoclonal antibodies for 30 min at 4°C and then washed twice and fixed with 0.1% paraformaldehyde before analysis. For indirect immunofluorescence, after the primary antibody incubation for 30 min at 4°C, 50 μl of goat anti-mouse fluorescein conjugate were added for 30 min, followed by washing and fixing. In some samples, whole blood was analyzed by direct immunofluorescence using the Q prep method (Coulter). Ten thousand cells were analyzed on either an EPICS C or an EPICS profile (Coulter) flow cytometer. Blasts were gated for analysis. Samples containing greater than 25% positive cells (using an isotype matched control Ig to designate the upper limit of negative fluorescence intensity) were scored as positive.

Radioimmunoassays. M195 IgG2a was purified by protein A affinity chromatography, radiolabeled with iodine-125, and used in direct radioimmunoassays on live leukemia and bone marrow cells as described before (16). M195 was labeled to 2–10 μCi/μg protein. Specific binding was determined by subtracting the amount of M195 IgG2a bound in the presence of an excess of unlabeled M195 IgG2a. Nonspecific binding was about 400 pg per million cells (1600 sites per cell). Binding at this level or below was therefore considered insignificant.

Morphological Designation of Leukemias. Acute leukemias in patients on the Leukemia Service at Memorial Hospital were classified according to the French-American-British (FAB) criteria (19) and were reviewed by at least one of the authors. Undifferentiated cells with negative histochemical stains which did not appear to be lymphoid and which did not meet FAB criteria for other diagnoses were classified as M0 (two cases only). Bone marrow aspirates and peripheral blood smears were stained with McNeil's tetrachrome (Polyscience, Warrington, PA) for morphology. Histochemical analysis included staining with Sudan black B and/or peroxidase and periodic acid Schiff, alpha-naphthylbutyrate and ASD chloroacetate esterase, acid phosphatase, terminal deoxynucleotidyl transferase (Tdt). Potential B cell neoplasms were analyzed by mouse red cell rosetting and by indirect immunoperoxidase for immunoglobulin products. The presence of the sheep

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red blood cell receptor on T cells was determined by ting at 37°C and 4°C (and by monoclonal antibody by flow cytometry).

Determination of Bone Marrow Colony-forming Units. Bone marrow mononuclear cells were assayed for colonies derived from CFU-GM, CFU-GEM, and BFU-E as described (13). Cultures consisted of Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) with 24% fetal calf serum, 0.8% deionized bovine serum albumin (Sigma Chemical, St. Louis, MO), 10^{-4} M 2-mercaptoethanol (Sigma Chemical), 1 U partially purified human urinary erythropoietin (49 U/mg) (Toyobo, NY, NY), 10% M0 T cell line conditioned media, and 1.3% methylcellulose. Cultures were prepared in quadruplicate and scored on days 7 or 14. In some assays, adherent cells were depleted first by plastic adherence at 37°C for 90 min.

Antibody mediated complement cytotoxicity of colony forming units was determined by incubating the marrow mononuclear cells first in excess monoclonal antibody (10–100 μg/ml) and low toxicity baby rabbit complement (Pel Freeze), at a final dilution of 1:8, for 30 min at 37°C followed by two washes with media. Alternatively, human serum was used as a complement source.

Preparation of Purified Normal Progenitor Blasts. Normal bone marrow cells were depleted of accessory and maturing cells to obtain enriched populations of progenitors by negative selection using density separations and a panel of monoclonal antibodies followed by immune rosetting or panning as described (13). The 12 antibodies reacted with cell surface antigens present on mature T, B, myeloid, and monocytic cells. Cells were then frozen in liquid N_2, and thawed once, then reseparated on Ficoll-Paque (Pharmacia, Piscataway, NJ) before use.

RESULTS

Distribution of M195 on Hematopoietic Neoplasms. The binding of mAb M195 to mononuclear cells from 227 patients as measured by flow cytometry is shown in Table 1. M195 was found on the majority of myeloblastic leukemias; 80% of the positive ANLL cases had greater than 50% of cells positive for M195. Forty percent of positive cases had greater than 75% of cells positive for M195. Lymphoid leukemias, lymphoproliferative disorders, and other nonmyeloid samples were virtually always negative (4% of cases positive).

A quantitative analysis of the total number of binding sites on several of the positive hematopoietic neoplasms was conducted by radioimmunoassay. Our accompanying study (16) showed that myelomonocytic leukemia cell lines expressed approximately 10,000 antigen sites per cell. The same quantity was seen on fresh ANLL cells from several patients (Fig. 1). Lymphoid leukemias and lym[illegible]mas, and chronic myelogenous leukemia (CML) cells in chronic phase did not express antigen on their surfaces.

The expression of M195 was compared to the FAB classification of ANLL (Table 2). M195 was expressed in all subclasses of ANLL except M0 and M6. Since there were very few leukemias of these two classes, the significance of this is not clear. However, when both the M0 and M1 classes were pooled, only 3 of 14 (23%) were positive compared to 30 of 44 M2, M3, or M4 leukemias (68%).

Tdt-positive ANLL (>320 ng/10^8 cells) also tended to be M195-negative (30%) compared to the Tdt-negative myeloid leukemias (74%). These data supported the suggestion from our earlier paper on cell lines that the M195 antigen was more highly expressed on early committed granulocytic precursor cells than on more undifferentiated earlier myeloid cells (16).

M195 was found on about a third of CML samples in chronic phase, all CML samples in myeloblastic or accelerated phase

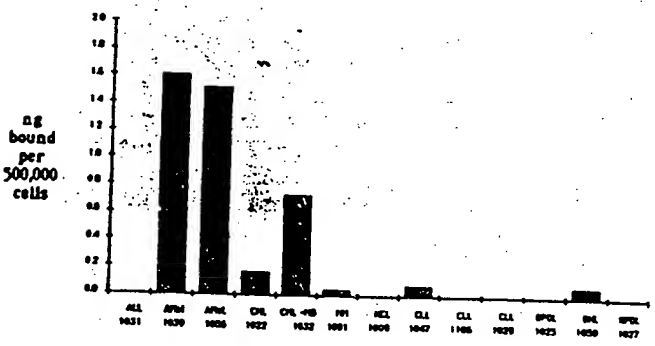


Figure 1. Direct radioimmunoassay for mAb M195 IgG on fresh hematopoietic neoplasms. The assay was conducted as described in Materials and Methods. The identities of the specimens are shown along the X axis; 1 ng bound per 500,000 cells is equivalent to 6000 IgG per cell. AMOL is acute monocytic leukemia; CML-MB is myeloblastic CML; MM is multiple myeloma. HCL is hairy cell leukemia; CLL is chronic lymphocytic leukemia; DPDL is diffuse poorly differentiated lymphoma; DHL is diffuse histiocytic lymphoma; NPDL is nodular poorly differentiated lymphoma. The lymphomas were suspensions made from lymph nodes. Background nonspecific binding was 0.2 ng bound. Only specific binding is shown.

Table 1. Distribution of M195 on Hematopoietic Neoplasms by Fow Cytometry[a]

	No. Tested	No. Positive	(%)
Acute nonlymphoblastic leukemia	54	34	(63)
Tdt-positive cases only	10	3	(30)
Chronic myelogenous leukemia—			
Accelerated and myeloblastic phase	7	7	(100)
Total myeloid, blastic cases	61	41	(67)
Chronic myelomonocytic leukemia	3	3	(100)
Myelodysplastic syndromes	25	12	(40)
Chronic myelogenous leukemia (chronic)	17	7	(41)
Acute lymphoblastic leukemia:			
Calla +	33	4	(12)
Calla −	8	0	
T-ALL	5	0	
Chronic myelogenous leukemia—			
Lymphoblastic phase	5	0	
Total lymphoid, blastic cases	51	4	(8)
Lymphoproliferative disorders (T + B)	19	1	(5)
Normal, nondiagnostic, and other	51	0	
Total cases	227		

[a]Conducted as described in the Materials and Methods.

Table 2. Distribution of M195 Antigen on Subgroups of AML

FAB Group	No. Tested	No. M195-Positive	(%)
M0	2	0	(0)
M1	12	3	(25)
M2	24	15	(63)
M3	5	5	(100)
M4	15	10	(67)
M5a	5	3	(60)
M5b	18	8	(44)
M6	2	0	(0)
M7	1	1	(100)

but not on lymphoblastic CML cells (Table 3A). Four of 46 acute lymphocytic (ALL) leukemias were M195-positive (Table 3B). These ALL samples were CALLA-positive pre-B leukemias. The total number of cells positive for M195 in these samples was rather low: 26%, 32%, 39%, and 42%. Other markers are shown for comparison. MY9 was present on five pre-B leukemias, with 27%, 28%, 35%, 35%, and 62% MY9-positive cells; four of these were different from those that were M195-positive. One case was 28% MY9-positive and 42% M195-positive. MY9 was also found on one of five cases of lymphoblastic CML (Table 3A).

A Comparison of M195 with CD33 Antigens. Our previous paper suggested that the distribution of M195 appeared similar to that described for CD33-reactive antibodies MY9 and L4F3. The protein target of M195 has thus far eluded detection (16). A comparison of M195 reactivity to other well characterized myeloid markers on the same leukemias is shown in Table 4 (this table does not include 30 leukemias that were not characterized by other markers that were included in Table 2). MY10, MY7, and MY4 were distributed among all subtypes in patterns dissimilar to M195. MY9 was strikingly similar to M195 in its pattern of distribution. An analysis of the concordance of M195 and MY9 in the flow cytometric studies on fresh, acute blastic leukemias (lymphoid and nonlymphoid) is shown in Table 5. In 93 cases of ANLL or acute lymphoid leukemias, either both markers were positive or both were negative. In 19 cases the binding differed, resulting in a concordance rate of 83% overall. This high, but not complete, concordance suggested that the M195 antigen might be related to or coexpressed with the CD33 antigen.

Cross-blocking experiments using iodine-125-labeled M195 IgG or F(Ab)'2 binding to HL60 leukemia cells in the presence of excess concentrations of various immunoglobulins are shown in Figure 2. Both MY9 and L4F3 (CD33-reactive), as well as the original M195 IgG, blocked binding of the ^{125}I-M195. MY7 (CD13), M31 (CD15), and OKB7 (CD21) did not inhibit binding. These data further confirmed the association between the M195 antigen and CD33 antigens. In other experiments (not shown) excess unlabeled M195 was able to block binding of about 50% of FITC-labeled MY9 to HL60 cells as measured by flow cytometry.

MAb M195 was also tested by Dr. T. Look (St. Jude, Memphis, TN) for reactivity with NIH-3T3 cells transfected with the DNA from myeloid cells and expressing the CD33 antigen (20). Both L4F3 (CD33) and MY9 (CD33) are reactive with these cells; M195 was reactive as well. This result, when taken in context with the nonidentical concordance data shown above, suggested that the M195 antigen was carried on the p67 (CD33) but was not the same as the previously described CD33 antigen epitopes recognized by L4F3 and MY9.

Diagnostic Utility of M195. MY9 is widely regarded as the standard marker for ANLL (21, 22). We compared the diagnostic utility of MY9 with M195 either alone or together on 81 blastic leukemias of either myeloid or lymphoid origin (Table 6). Eighty-four percent of ANLL expressed either M195 or MY9, but each antibody alone failed to identify more than a quarter of cases. Among lymphoid cases either MY9 or M195 was occasionally expressed, but both antibodies were expressed together only once. In this case the reactivity was weak: 28% MY9 + and 42% M195 +. Thus, the presence of both M195 and MY9 positivity on a leukemia sample had 98% specificity in defining that leukemia as ANLL.

Table 4. Immunophenotype of FAB Subgroups of AML at Memorial Hospital*

Group	No. Tested	% Testing Positive for:				
		My10	My7	My9	M195	My4
M0	2	100	100	0	0	0
M1	5	80	50	60	40	0
M2	15	67	33	73	73	7
M3	4	100	50	100	100	75
M4	10	70	40	80	70	44
M5a	3	100	33	100	67	0
M5b	12	58	45	75	58	17
M6	2	50	100	50	0	0
M7	1	100	0	100	100	0

*Conducted as described in Materials and Methods.

Table 3A. Immunophenotype of Chronic Myelogenous Leukemia at Memorial Hospital*

	No. tested	% Testing Positive for:						
		M195	My9	My10	My7	My4	Calla/B4	Ia
CML chronic phase	17	41	41	19	18	6	6	24
CML accelerated and myeloblastic	7	100	70	100	86	28	ND	86
CML lymphoblastic	5	0	20	100	50	0	80	80

Table 3B. Immunophenotype of Acute Lymphoblastic Leukemias at Memorial Hospital*

ALL type	No. Tested	% Testing Positive for:					
		Ia	My10	B4	B1	M195	My9
CALLA +	33	82	58	91	52	12	15
CALLA −	8	88	88	100	0	0	0
T-ALL	5	0	0	0	0	0	0
Total non-T ALL	41	83	64	95	41	10	12
Total ALL	46	74	57	85	37	9	11

*Conducted as described in Materials and Methods.

Table 5. Concordance Data for M195 and My9 among 112 Blastic Leukemias

Reactivity Pattern	No. of Cases
My9 + and M195 +	38
My9 − and M195 −	55
My9 + and M195 −, or My9 − and M195 +	19
Overall concordance	83%

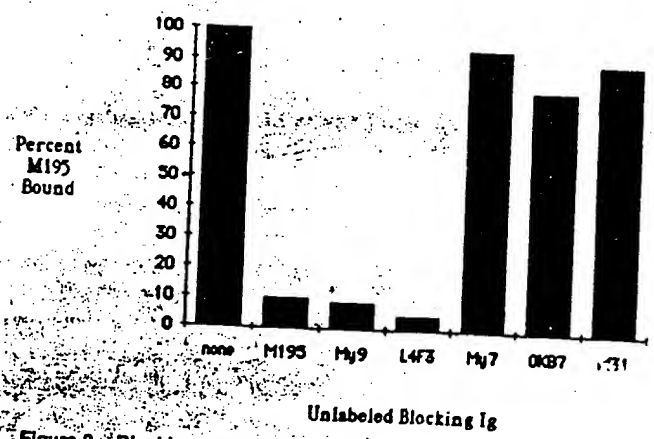


Figure 2. Blocking of M195 direct radioimmunoassay by excess unlabeled Ig. A 50–100-fold molar excess of the antibodies designated along the X axis were added to HL60 target cells followed by ^{125}I-M195 at 4°C for 60 min. The amount of bound ^{125}I-M195 is shown on the Y axis. Binding of M195 without competing IG was normalized to 100%.

Expression of M195 on Hematopoietic Colony-forming Cells. The expression of M195 on leukemia cells, but not on mature nonadherent peripheral blood cells nor on any detectable nonadherent bone marrow cells (16), suggested that M195 might be expressed on a small group of hematopoietic progenitors. The expression of M195 antigen on hematopoietic progenitors was studied by analyzing the recovery of bone marrow colonies after treatment of bone marrow mononuclear cells with M195 and rabbit complement (Table 7). Complement alone, antibody alone, and no antibody or complement treatments were used as negative controls. Antibody to human IA antigen (gift of Dr. J. D. Griffin) was used as a positive control. The number of CFU-GEMM recovered was not sufficient to obtain statistically significant data. In three of four experiments, M195 and complement eliminated almost all of the 14-day CFU-GM; burst forming unit erythrocytes were also killed, although the average recovery was somewhat higher.

In order to determine the extent of expression of M195 on early hematopoietic progenitors, radioimmunoassays were conducted on highly purified blasts. The cells used were isolated by negative selection with a panel of 12 monoclonal antibodies and immunorosetting or panning followed by freezing and thawing once (13). These cells are morphologically blasts and represent a progenitor cell population 50–100-fold purer than bone marrow mononuclear cells. Five to fifteen percent of these cells typically form myeloid and erythroid colonies.

No binding of ^{125}I-M195 above background was found in testing three different samples of these normal, early blast cells. A small percentage of positive cells could escape detection using this assay.

Because these data suggested that M195 antigen was expressed on a minor population of bone marrow cells responsible for CFU-GM colonies, we attempted to identify these cells by positive selection with panning, immunomagnetic bead separation, affinity sepharose bead separation, and fluorescence-activated cell sorting. None of these methods selected out a M195-positive subpopulation. This may be due to weak antigen expression, antibody affinity, or other unknown problems.

Effects of M195 on Bone Marrow Progenitors in the Presence of Human Complement. Because we anticipated possible use of M195 in vivo for therapy of ANLL, we studied the effects of M195 on CFU-GM and 5FU-E from normal bone marrow in the presence of human serum as a complement source (Table 8A). No killing of CFU-GM or BFU-E was seen at 14 days. The effect of the continued presence of M195 in bone marrow culture was also studied by adding the antibody to the methyl cellulose at days 1 and 5 after plating, with no added complement (Table 8B). These experiments were done to determine if the antibody had a growth stimulatory or inhibitory effect on progenitor cells in the marrow. No effects were seen. Similar growth studies of peripheral blood mononuclear cells and HL60 leukemia cells were also negative.

DISCUSSION

This paper describes the distribution of mAb M195's binding on fresh leukemia cells and early hematopoietic progenitors. In our accompanying paper (16) we showed that the M195 antigen was present on myelomonocytic leukemia cells and a fraction of monocytes but was not detectable on more mature myeloid cells present in the bone marrow or peripheral blood nor on nonhematopoietic cells and tissues. In this paper we extend the description of the M195 antigen and directly compare it to other well-characterized myeloid and monocytic antigens. Among 227 fresh hematopoietic samples studied, M195 antigen expression was largely restricted to differentiated ANLL. Undifferentiated and Tdt-positive ANLL were less likely to display antigen. However, FAB classifications did not correlate specifically with M195 expression.

Quantitative analysis by radioimmunoassay showed that about 10,000 sites were expressed on the cell surface of ANLL cells. Our studies (16) have demonstrated rapid modulation of these sites after antibody binding.

Table 6. Diagnostic Utility of M195 and My9 among Blastic Leukemias

	Positive Cases with the Indicated Marker(s)			
	M195 Alone	My9 Alone	Both M195 and MY9	Either M195 or My9
Sensitivity[a] in 61 Myeloblastic cases	67%[b]	74%	67%	84%
Specificity[c] in 51 lymphoblastic cases	8%	12%	2%	20%

[a]Antibody(s) should be positive in all cases.
[b]Percent of cases positive by flow cytometry.
[c]Antibody(s) should be negative in all cases.

Table 7. ...ecovery Colonies after Treatment with Antibody and Complement

Treatment	% Recovery of the Following Colonies		
	Day 7 CFU-GM	Day 14 CFU-GM	BFU-E
Nil	124[a] (111, 136)[b]	126 (139, 104, 111, 110)	103 (143, 83, 115, 71)
M195 alone	108 (97, 120)	124 (126, 181, 93, 98)	107 (141, 114, 98, 74)
Complement alone	100[c]	100	100
M195 plus complement	6 (10, 1)	17 (0, 60, 3, 6)	33 (8, 77, 6, 40)
Anti-IA plus complement	0 (0, ND[d])	1 (0, 2, 0, ND)	6 (5, 11, 1, ND)

[a]Mean of all experiments shown.
[b]Percent recovery of an individual experiment.
[c]The "complement alone" treatment was considered to be 100% recovery, and other data on this chart were normalized to that value. Plating efficiency was between 0.10 and 0.15 percent.
[d]ND = Not determined.

Several antigens are currently used to diagnose ANLL by flow cytometry. Among these, CD33 antibodies, MY9 (5), and L4F3 or L1B2 (4), appear to be most widely and most specifically distributed on ANLL. M195 antigen was concordantly expressed with MY9 on 83% of cases. Moreover, although neither antigen was expressed on 100% of ANLL, the combination of both M195 and MY9 could be used to diagnose ANLL with 98% specificity if *both* were expressed on a leukemia sample. We are currently using this combination to aid in the diagnosis of acute leukemias at Memorial Hospital.

The close coexpression of M195 and MY9 suggested that M195 might bind to the CD33 protein target [p67] (20). Efforts to identify the M195 target have been unsuccessful (16). Blocking experiments shown here demonstrated probable identity of the M195 target with the CD33 protein. Moreover, binding of M195 to CD33 DNA transfectants was shown. Despite these data, since flow cytometry data showed nonidentical concordance with MY9, it is likely that M195 does not bind to the same CD33 epitope as MY9 or L4F3.

Although M195 antigen was found on a greater percentage of ANLL samples of the FAB classifications M2, M3, and M4 than on other types, the presence of M195 binding could not be used to predict morphology or vice versa. Other studies comparing immunophenotype with morphologic phenotype have come to similar conclusions since there was considerable overlap of markers into each type of ANLL (23–25). Some discrimination of monocytic from myeloid ANLL has been shown (22, 26), however.

The CD33 antigen is expressed on early myelomonocytic progenitor cells (4, 5), but not on the ultimate progenitors (17).

Table 8A. Effects of M195 on Hematopoietic Stem Cells in the Presence of Human Complement[a]

Treatment	No. of Colonies, Day 14	
	CFU-GM	BFU-E
None	116 (100%)[b]	154 (100%)
Complement (C') alone	102 (100%)	121 (100%)
M195	132 (113%)	158 (102%)
M195 + C'	141 (138%)	127 (105%)

Table 8B. Effect of the Continued Presence of M195 on Colony Forming Cells[c]

Treatment	No. of colonies, Day 14	
	CFU-GM	BFU-E
Nil	125 (100%)	188 (100%)
M195	124 (99%)	168 (89%)

[a]Human serum was added at a final dilution of 1/6 as described for rabbit complement in Materials and Methods.
[b]Quadruplicate control plate results are normalized to 100%.
[c]M195 IgG was added directly o growing cultures as described in Materials and Methods.

This restriction has allowed selective purging of ANLL cells from bone marrow while still permitting regrowth of normal cells in selective cases (18). This avenue of therapy is being pursued elsewhere. M195, as expected, was expressed on CFU-GM and to a lesser extent on BFU-E. Since, like MY9 and L4F3, M195 readily kills cells with rabbit complement, it may be useful as a purging agent in ANLL. Studies of this application are underway (E. Berman, unpublished data).

Radioimmunoassays with M195 on highly purified early blasts did not detect significant antigen expression. Because the radioimmunoassay could miss M195 expression on small subpopulations within this group of cells, long-term marrow cultures will need to be done to help further define and confirm this finding. Based on the data here and in our accompanying paper (16), the distribution of the M195 antigen among hematopoietic differentiation appears similar to that described for other CD33 antigens (4, 5). This includes early committed myeloid progenitors, but perhaps not the earliest colony forming cells (17, 18).

The M195 antigen is not expressed on adult human tissues. Therefore, in addition to its use as a diagnostic marker of ANLL and as a purging agent, M195 can potentially be used as a therapeutic agent in vivo. Since the antibody does not have *in vitro* cytotoxic effects alone or in the presence of human serum as a complement source, it is not likely to cause lysis of ANLL cells. However, upon binding of mAb M195, the antibody is rapidly internalized (to be published elsewhere), and thus the application of mAb M195 as a carrier of toxins or alpha-emitting isotopes to ANLL cells in vivo may be feasible.

Acknowledgments. The authors thank Ms. Carol Turner and Ms. Linda Forte for assistance in the preparation of this manuscript. The binding of M195 to CD33 transfectants by Dr. Thomas Look is greatly appreciated. This work was supported in part by the National Institutes of Health Grant CA08748, the Jennie R. and Oliver S. Donaldson Charitable Trust, and the Westvaco Fund.

REFERENCES

1. Civin CI, Mirro J, Banquerigo ML. My-1, a new myeloid-specific antigen identified by a mouse monoclonal antibody. Blood 1981;57:842–845.
2. Griffen JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 1981;68:932–941.
3. Perussia B, Trichieri G, Lebman D, Jankiewicz J, Lange B, Rovera G. Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. Blood 1982;59:382–392.
4. Andrews RG, Torok-Storb B, Bernstein ID. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood 1983;62:124–132.

5. Griffin JD, Linch D, Sabbath K, Larcom P, [illegible]oss[illegible]F. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 1984;8:521–534.
6. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 1984;133:157–165.
7. Katz FE, Tindle R, Sutherland DR, Greaves MF. Identification of a membrane glycoprotein associated with hematopoietic progenitor cells. Leuk Res 1985;9:191–198.
8. Askew DS, Eaves AC, Takei F. NHL-30.5: a monoclonal antibody reactive with an acute myeloid leukemia (AML)-associated antigen. Leuk Res 1985;9:135–145.
9. Ferrero D, Gabbianelli M, Peschle C, Lange B, Rovera G. Surface phenotypes of human hematopoietic progenitor cells defined by monoclonal antibodies. Blood 1985;66:496–502.
10. Drexler HG, Sagawa K, Menon M, Minowada J. Reactivity patterns of myeloid monoclonal antibodies with emphasis on MCS-2. Leuk Res 1986;10:17–23.
11. Peng R, Al-Katib A, Knowles DM, Lu L, Borxmeyer H, Tolidjian B, Chiao J-W, Koziner B, Wang CY. Preparation and characterization of monoclonal antibodies recognizing two distinct differentiation antigens (Pro-Im1, Pro-Im2) on early hematopoietic cells. Blood 1984;64:1169–1178.
12. Wisniewski D, Knowles R, Wachter M, Strife A, Clarkson B. Expression of two natural killer cell antigens, H-25 and H-366, by human immature myeloid cells and by erythroid and granulocytic/monocytic colony-forming units. Blood 1987;69:419–429.
13. Strife A, Lambek C, Wisniewski D, Gulati S, Gasson JC, Golde DW, Welte K, Gabrilove JL, Clarkson B. Activities of four purified growth factors on highly enriched human hematopoietic progenitor cells. Blood 1987;69:1508–1523.
14. Ball ED, Mills LE, Coughlin CT, Beck R, Cornwell GG. Autologous bone marrow transplantation in acute myelogenous leukemia: *in vitro* treatment with myeloid cell-specific monoclonal antibodies. Blood 1986;68:1311–1315.
15. Ball ED, Bernier GM, Cornwell GG, McIntyre OR, O'Donnell JF, Fanger MW. Monoclonal antibodies to myeloid differentiation antigens: *in vivo* studies of three patients with acute myelogenous leukemia. Blood 1983;62:1203–1210.
16. Tanimoto M, Scheinberg DA, Cardo CC, Huie D, Clarkson BD, Old LJ. Restricted expression of an early myeloid and monocytic cell surface antigen defined by monoclonal antibody M195. Leukemia 1989;3:339–348.
17. Andrews RG, Takahashi M, Segal GM, Powell JS, Bernstein ID, Singer JW. The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. Blood 1986;68:1030–1035.
18. Bernstein ID, Singer JW, Andrews RG, Keating A, Powell JS, Bjornson BH, Cuttner J, Najfeld V, Reaman G, Raskind W, Sutton DMC, Fialkow PJ. Treatment of acute myeloid leukemia cells *in vitro* with a monoclonal antibody recognizing a myeloid differentiation antigen allows normal progenitor cells to be expressed. J Clin Invest 1987;79:1153–1159.
19. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick H, Sultan C. Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med 1985;103:626–629.
20. Peiper SC, Lemons RS, Ashmun RA, Look AT. DNA-mediated transfer of the gene encoding p67 (CD33), a myeloid differentiation antigen recognized by the MY9, L1B2, and L4F3 monoclonal antibodies. In: McMichael AJ, ed. Leukocyte typing III. Oxford: Oxford University Press, 1986;622–625.
21. Alvey PL, Greaves MF. A computer program for interpreting immunophenotypic data as an aid to the diagnosis of leukemia. Leukemia 1987;1:527–540.
22. Neame PB, Soamboonsrup P, Browman GP, Meyer RM, Bender A, Wilson WEC, Walker IR, Saeed N, McBride JA. Classifying acute leukemia by immunophenotyping: a combined FAB-immunologic classification of AML. Blood 1986;68:1355–1362.
23. Pessano S, Palumbo A, Ferrero D, Pagliardi GL, Bottero L, Lai SK, Meo P, Carter C, Hubbell H, Lange B, Rovera G. Subpopulation heterogeneity in human acute myeloid leukemia determined by monoclonal antibodies. Blood 1984;64:275–281.
24. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Bevendga RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557–563.
25. Van der Reijden HJ, Van Rhenen DJ, Lansdorp PM, Van't Veer MD, Langenhuijsen MMAC, Engelfriet CP, Von dem Borne AFGK. A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 1983;61:443–448.
26. Linch DC, Allen C, Beverley PCL, Bynoe AG, Scott CS, Hogg N. Monoclonal antibodies differentiating between monocytic and nonmonocytic variants of AML. Blood 1984;63:566–573.

US005730982A

United States Patent [19]

Scheinberg

[11] Patent Number: 5,730,982

[45] Date of Patent: Mar. 24, 1998

[54] **THERAPEUTIC USE OF HYPERVARIABLE REGION OF MONOCLONAL ANTIBODY M195 AND CONSTRUCTS THEREOF**

[75] Inventor: **David A. Scheinberg**, New York, N.Y.

[73] Assignee: **Sloan-Kettering Institute for Cancer Research**, New York, N.Y.

[21] Appl. No.: **383,615**

[22] Filed: **Feb. 2, 1995**

Related U.S. Application Data

[63] Continuation of Ser. No. 56,957, May 3, 1993, abandoned, which is a continuation of Ser. No. 450,918, Dec. 14, 1989, abandoned.

[51] Int. Cl.[6] A61K 39/395; C07K 16/28

[52] U.S. Cl. **424/181.1**; 424/183.1; 530/388.22; 530/391.3; 530/391.5; 530/391.7; 530/391.9

[58] Field of Search 530/391.3, 391.7, 530/387.7, 388.7, 387.3, 388.22, 391.5, 391.9; 435/240.27; 424/178.1, 183.1, 144.1, 154.1, 155.1, 181.1

[56] **References Cited**

PUBLICATIONS

Scheinberg et al. Leukemia 3:440–445, 1989.

Tanimoto et al. Leukemia 3:339–348, 1989.

Andrews et al. Blood 62:124–132, 1983.

Bernstein et al. J. Clin Invest. 79:1153–1159, 1987.

Shawler et al. Cancer Res. 44:5921–5927, 1984.

Peiper et al. pp. 622–625 in Leulcocyte Typing III, MaeMichael et al. Ed. Oxford Univ. Press 1986.

Houghton et al. Seminars in Clinical Oncology 13: 165–179 1986.

Morrison Science 229:1202–1207, 1983.

Waldman Science 252:1657–1662, 1991.

Blakey et al., "Monoclonal Antibody Therapy Prog Allergy", H. Waldmann Ed., Basel Karger 1988, vol. 45, pp. 50–90.

Engleman et al., Eds. "Human Hybridomas and Monoclonal Antibodies", Plenum Press, 1985, pp. 160–161.

Primary Examiner—Lila Feisee
Assistant Examiner—Nancy A. Johnson
Attorney, Agent, or Firm—John P. White

[57] **ABSTRACT**

Therapeutic agents and methods for treating and diagnosing acute or chronic leukemia are provided. Such agents comprises monoclonal antibody M195, or a chimeric antibody containing the hypervariable region of M195, conjugated to a cytotoxic agent, e.g. a radioisotope.

21 Claims, 17 Drawing Sheets

FIG. 1A

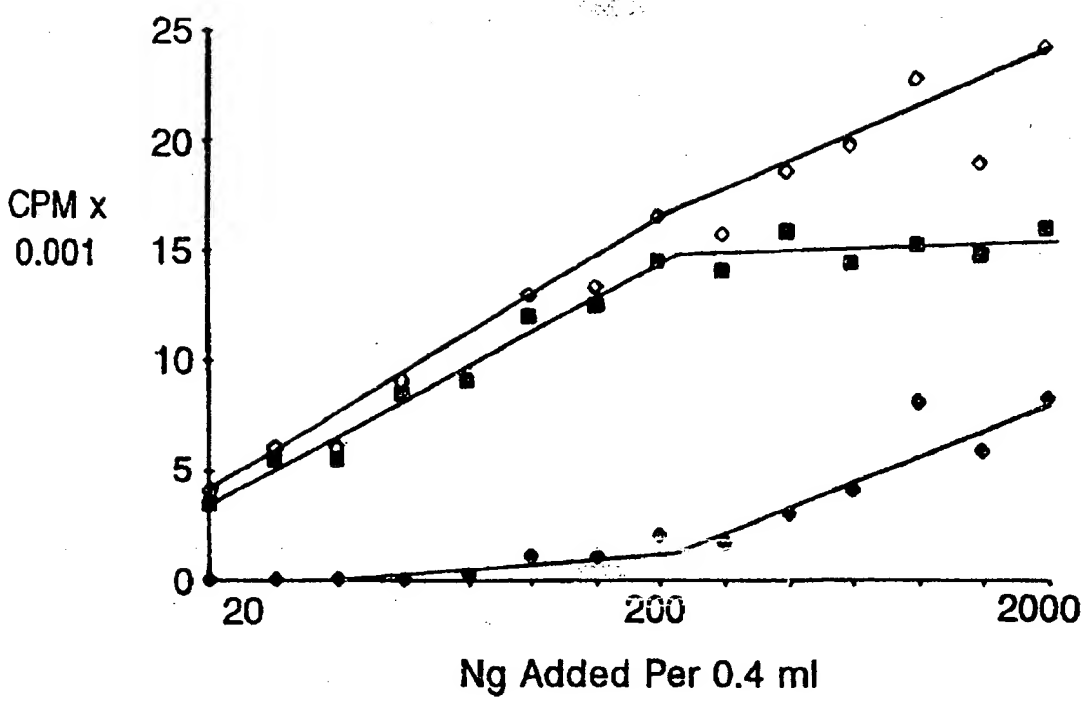


FIG. 1B

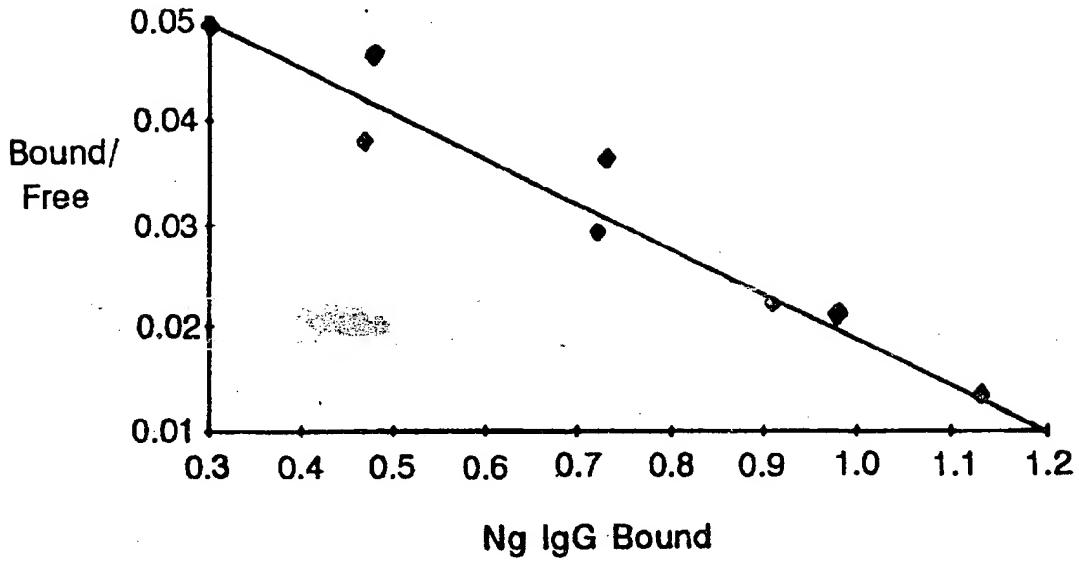

0.05
0.04
0.03
0.02
0.01
Bound/
Free
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
Ng IgG Bound

FIG. 1C

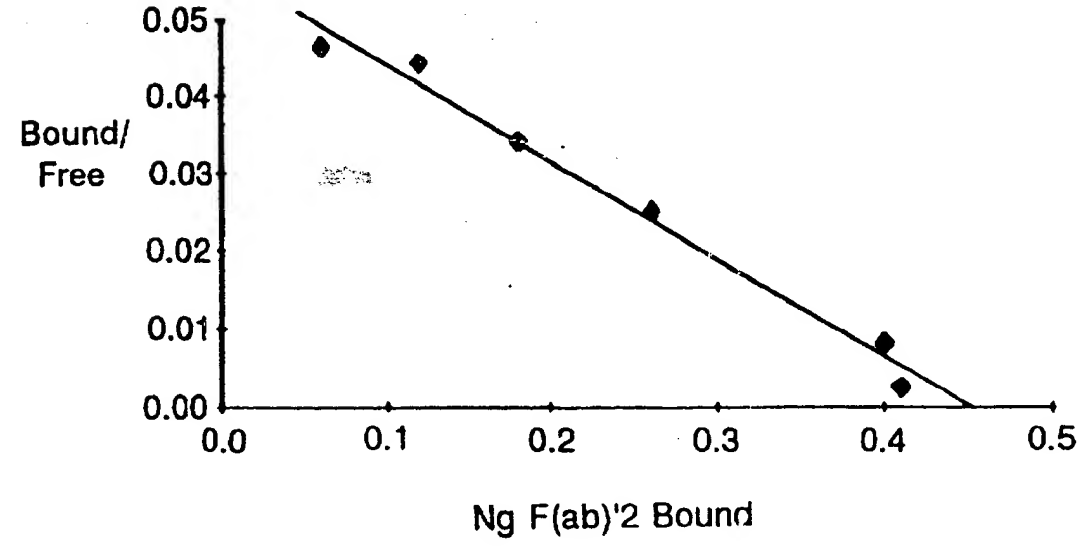

0.05
0.04
0.03
0.02
0.01
0.00
Bound/
Free
0.0
0.1
0.2
0.3
0.4
0.5
Ng F(ab)'2 Bound

FIG. 2

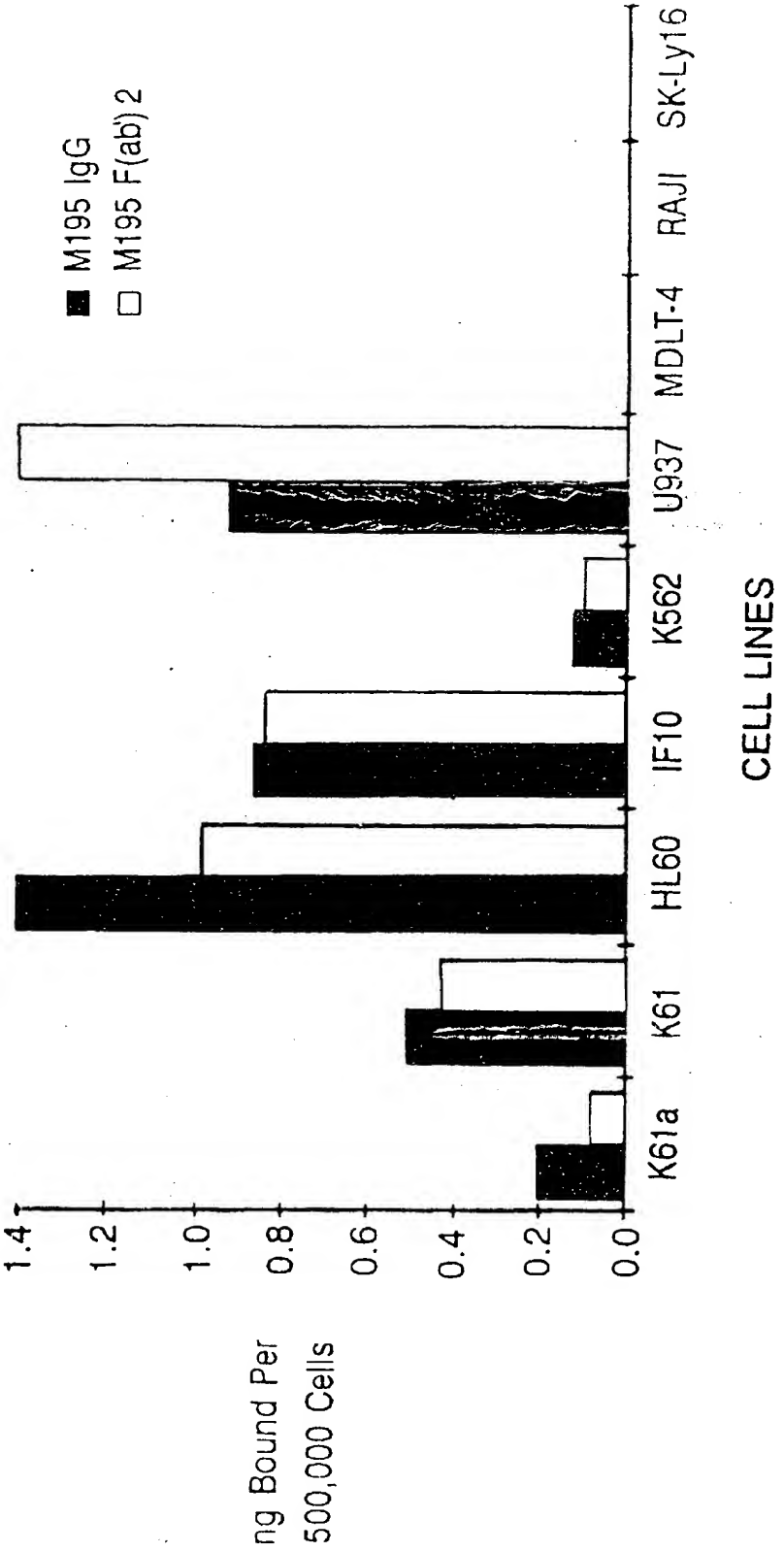

M195 IgG
M195 F(ab') 2
ng Bound Per 500,000 Cells
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
K61a
K61
HL60
IF10
K562
U937
MDLT-4
RAJI
SK-Ly16
CELL LINES

FIG. 3

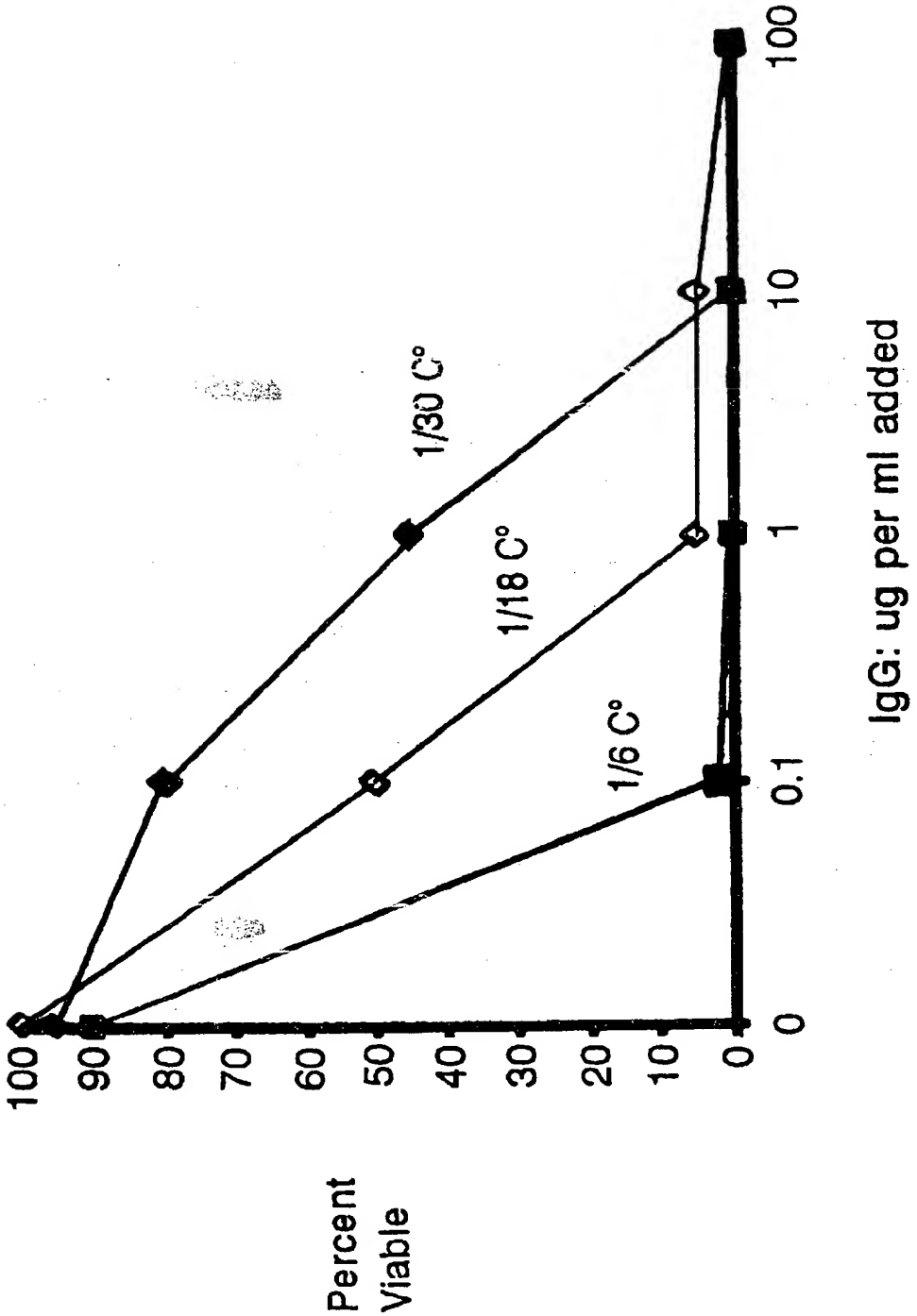
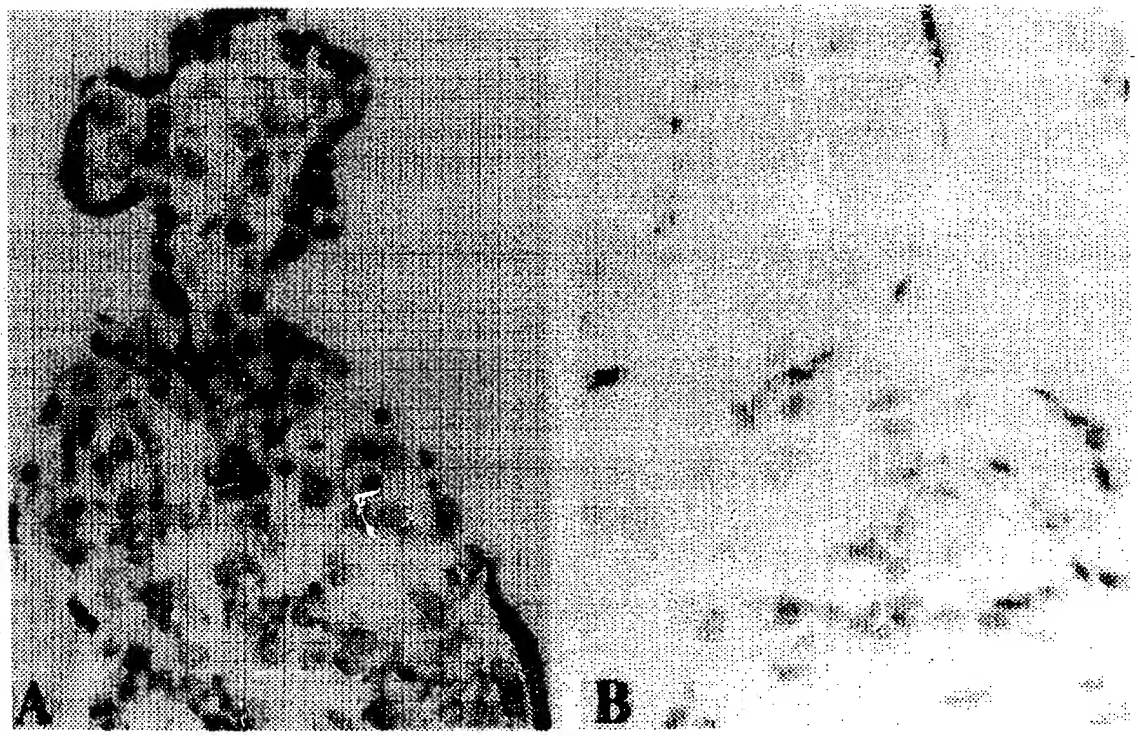

1/30 C°
1/18 C°
1/6 C°
100
90
80
70
60
50
40
30
20
10
0
Percent Viable
0
0.1
1
10
100
IgG: ug per ml added

FIG. 4A

FIG. 4B



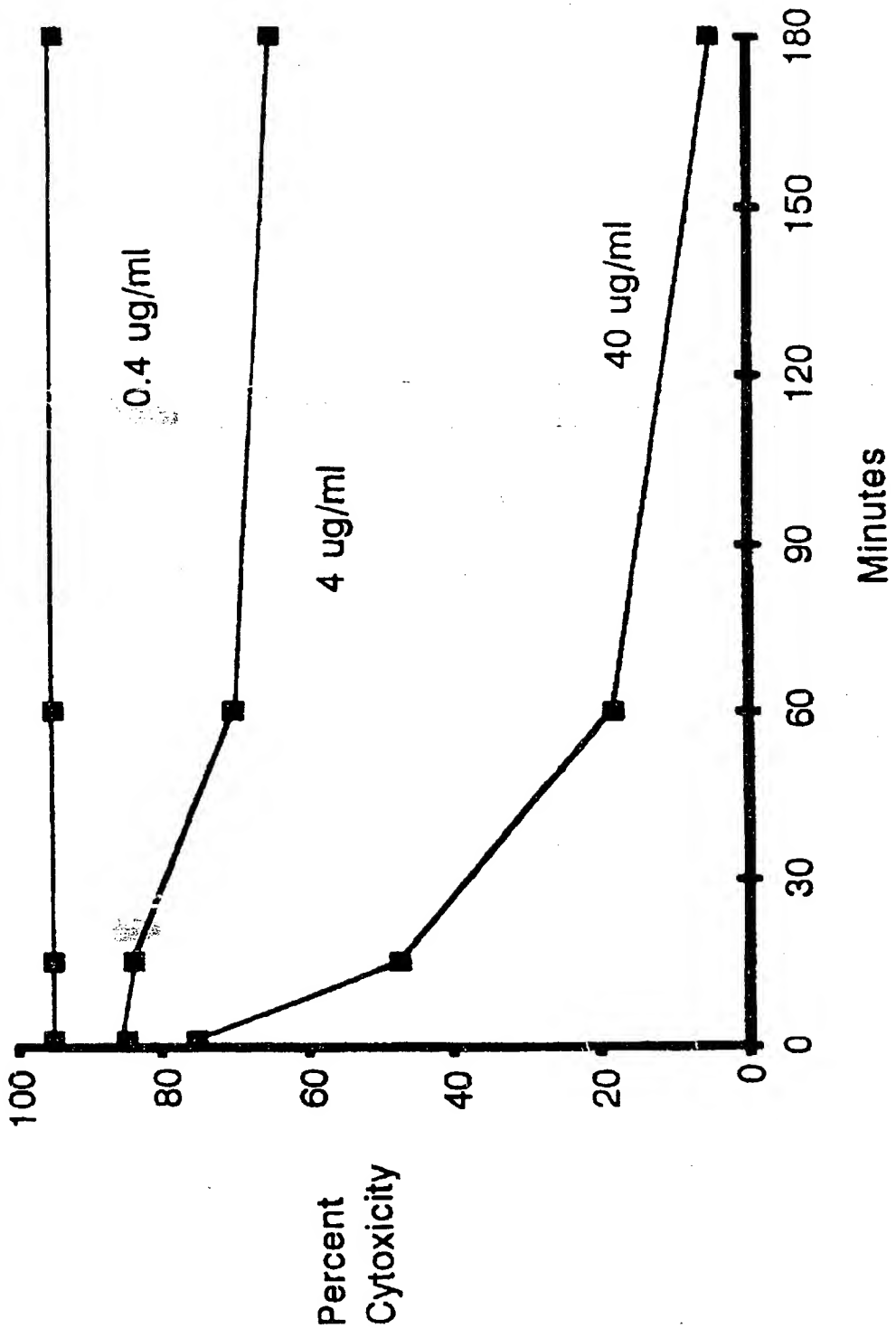
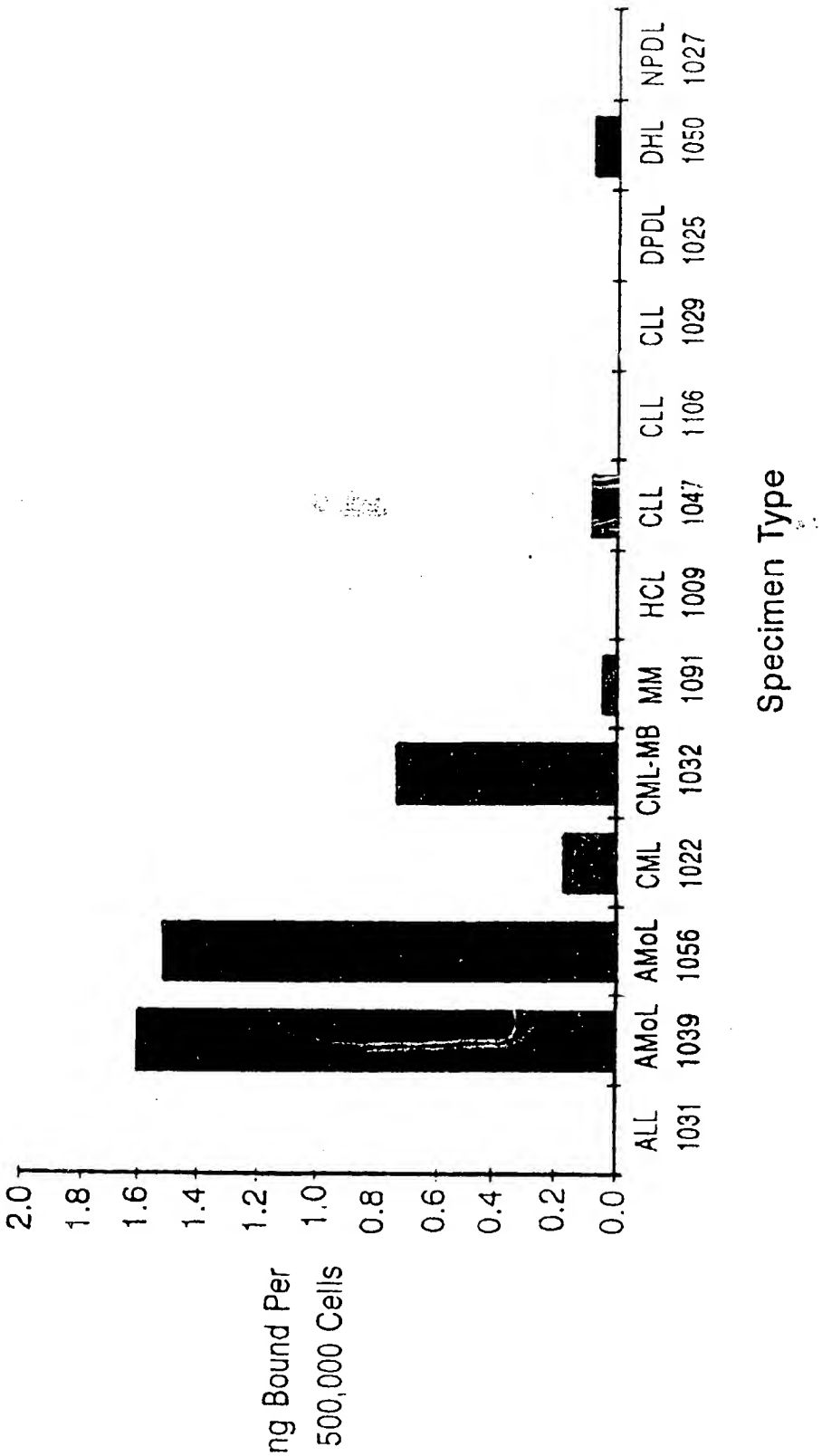

0.4 ug/ml
4 ug/ml
40 ug/ml
100
80
60
40
20
0
Percent Cytoxicity
0
30
60
90
120
150
180
Minutes

FIG. 5

FIG. 6


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ng Bound Per 500,000 Cells
ALL 1031
AMoL 1039
AMoL 1056
CML 1022
CML-MB 1032
MM 1091
HCL 1009
CLL 1047
CLL 1106
CLL 1029
DPDL 1025
DHL 1050
NPDL 1027
Specimen Type

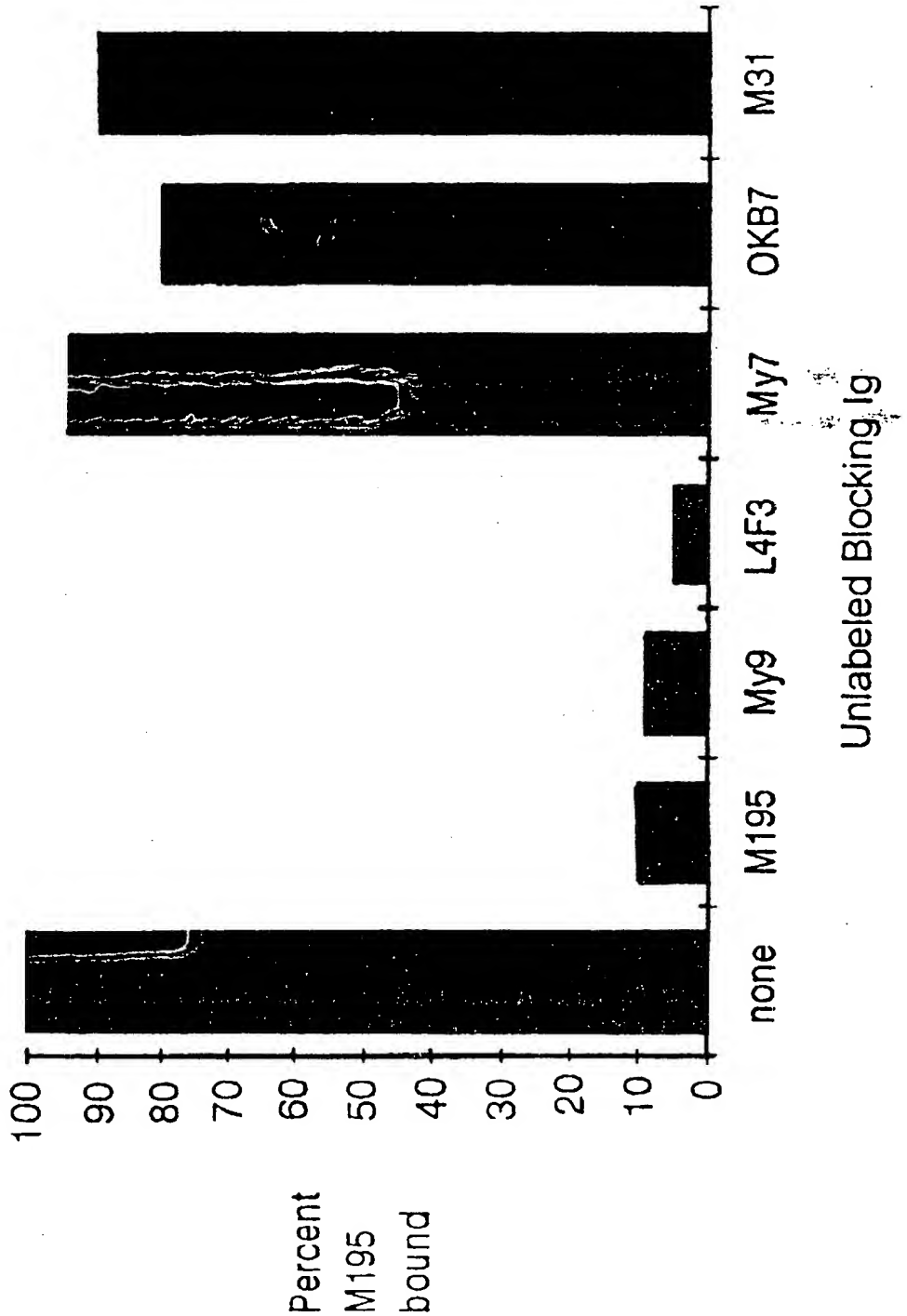

Percent M195 bound
100
90
80
70
60
50
40
30
20
10
0
none
M195
My9
L4F3
My7
OKB7
M31
Unlabeled Blocking Ig

FIG. 7

FIG. 8A

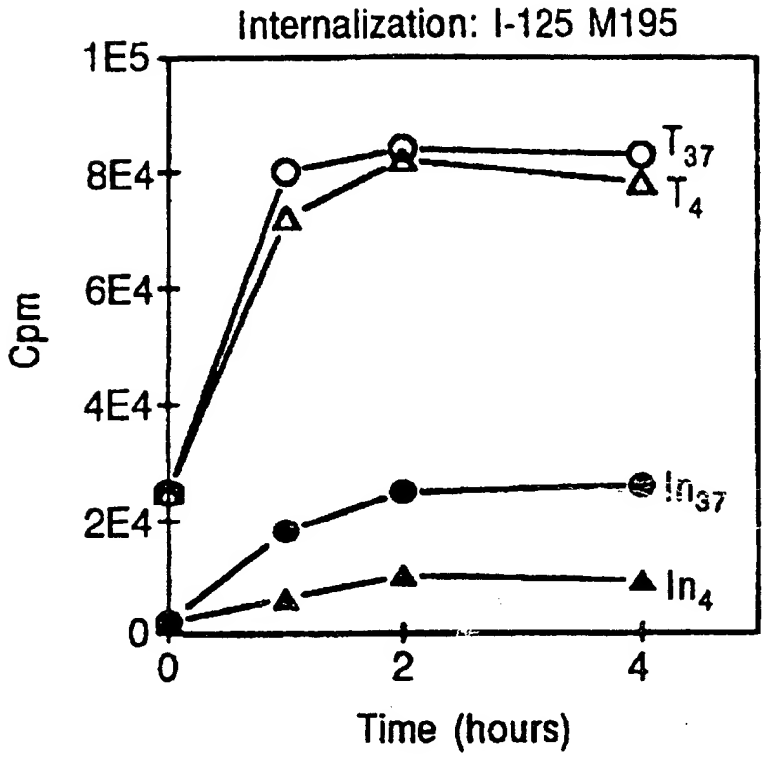

Internalization: I-125 M195
1E5
8E4
6E4
4E4
2E4
0
Cpm
T_{37}
T_4
In_{37}
In_4
0
2
4
Time (hours)

FIG. 8B

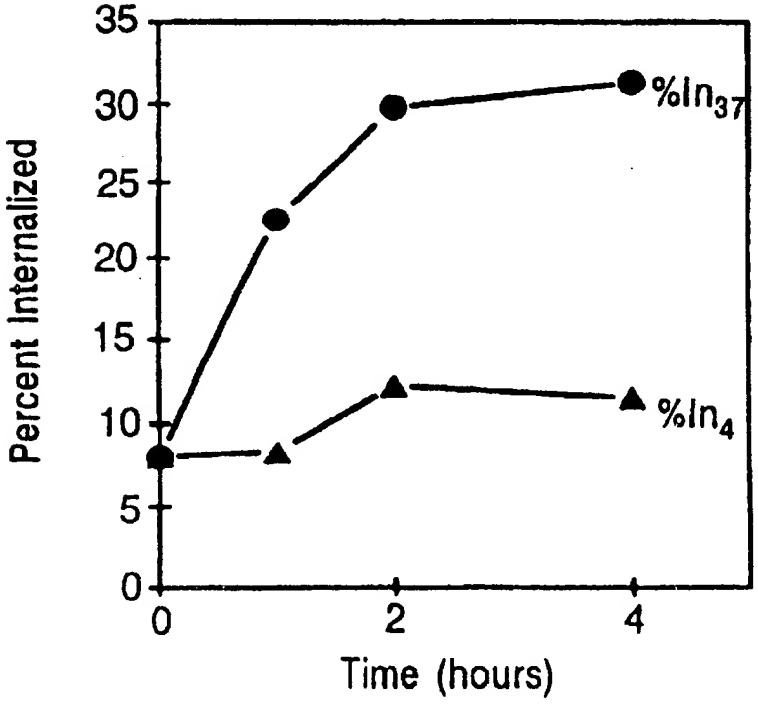

35
30
25
20
15
10
5
0
Percent Internalized
$\%In_{37}$
$\%In_4$
0
2
4
Time (hours)

FIG. 8C

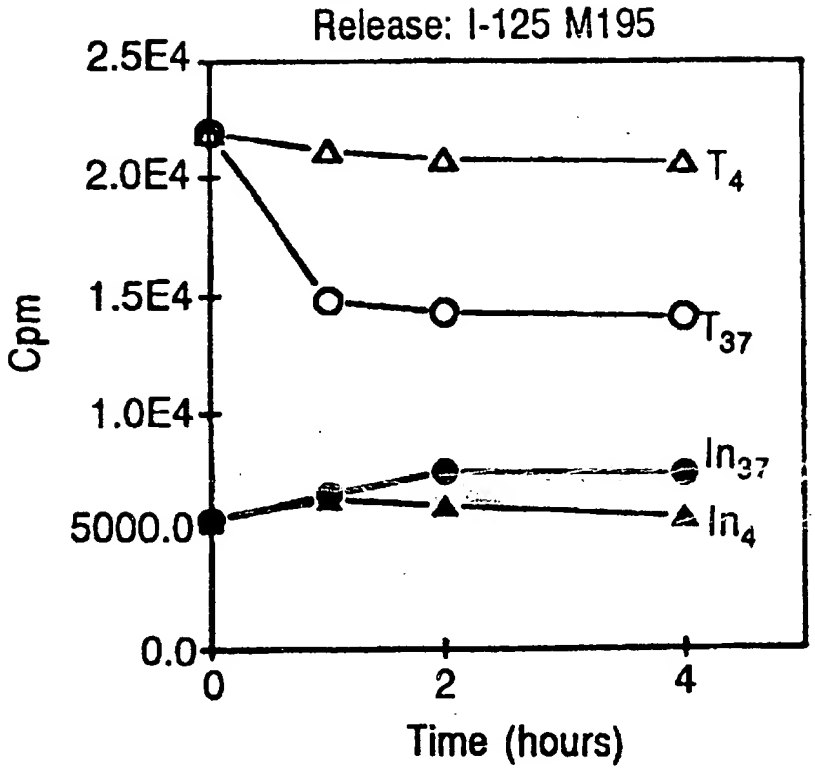

Release: I-125 M195
2.5E4
2.0E4
1.5E4
1.0E4
5000.0
0.0
Cpm
T_4
T_{37}
In_{37}
In_4
0
2
4
Time (hours)

FIG. 8D

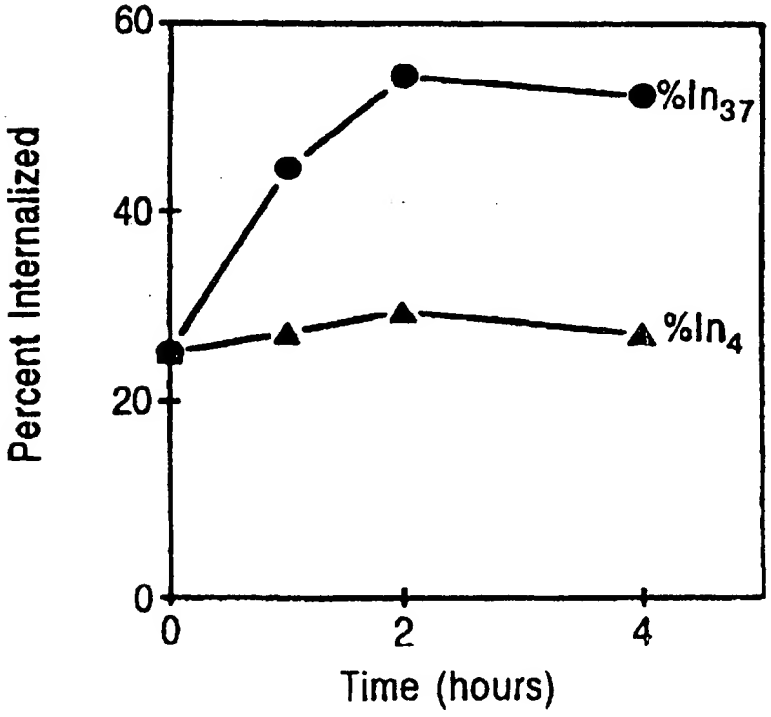

60
40
20
0
Percent Internalized
$\%In_{37}$
$\%In_4$
0
2
4
Time (hours)

FIG. 9A

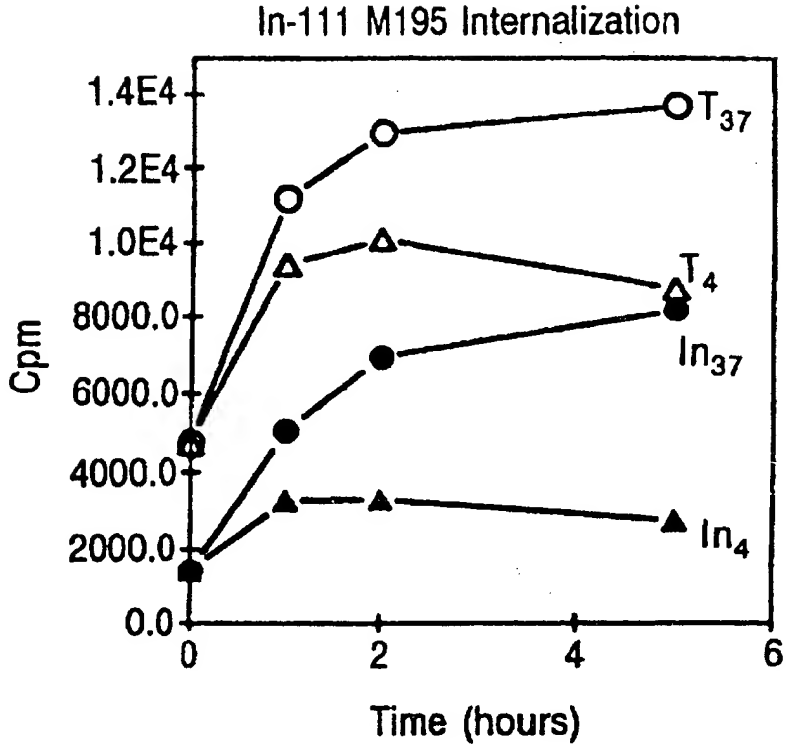

In-111 M195 Internalization
1.4E4
1.2E4
1.0E4
8000.0
6000.0
4000.0
2000.0
0.0
Cpm
0
2
4
6
Time (hours)
T_{37}
T_4
In_{37}
In_4

FIG. 9B

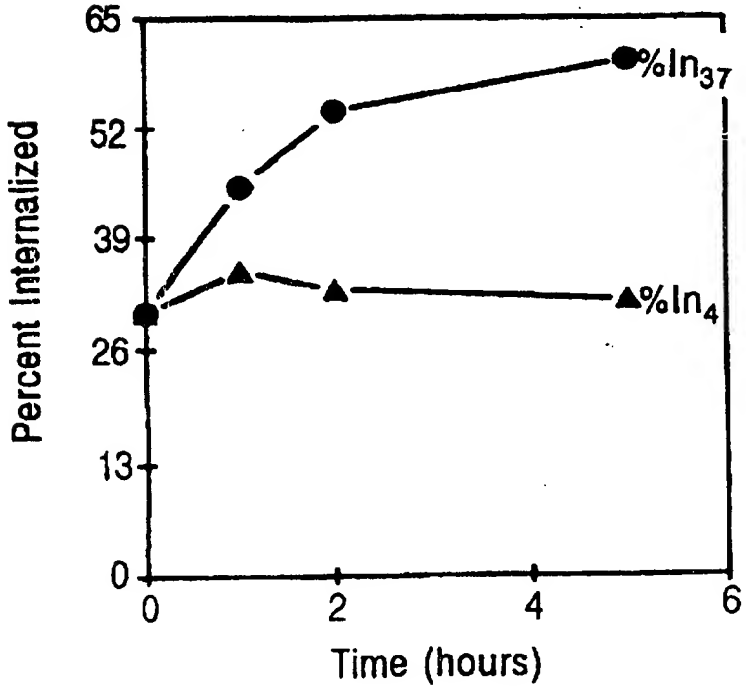

65
52
39
26
13
0
Percent Internalized
0
2
4
6
Time (hours)
%In_{37}
%In_4

FIG. 9C

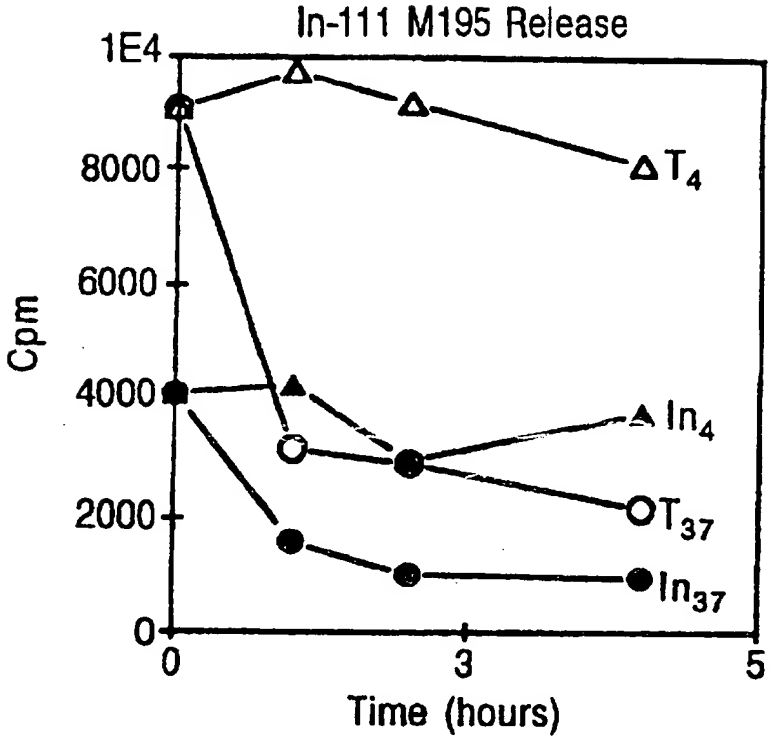

In-111 M195 Release
1E4
8000
6000
4000
2000
0
Cpm
T_4
In_4
T_{37}
In_{37}
0
3
5
Time (hours)

FIG. 9D

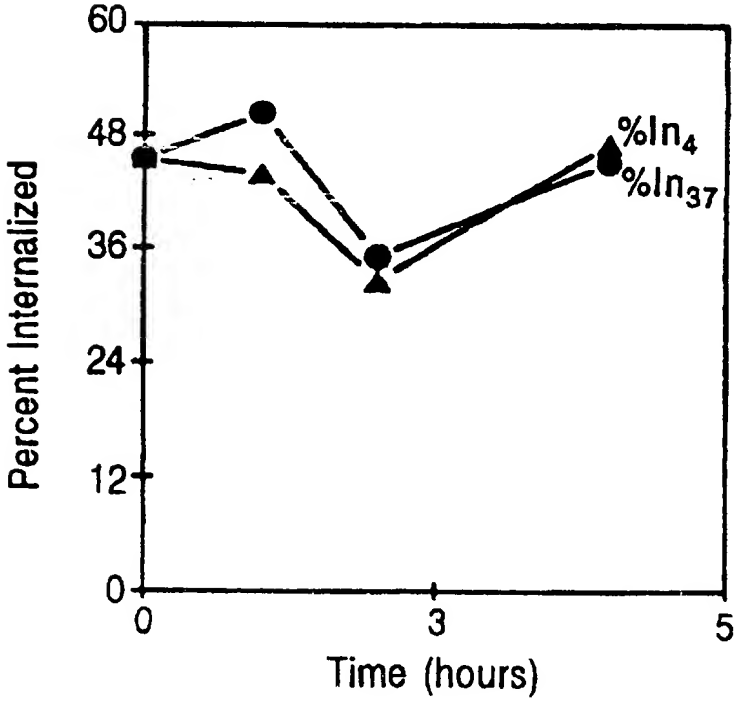

60
48
36
24
12
0
Percent Internalized
%In_4
%In_{37}
0
3
5
Time (hours)

FIG. 10A

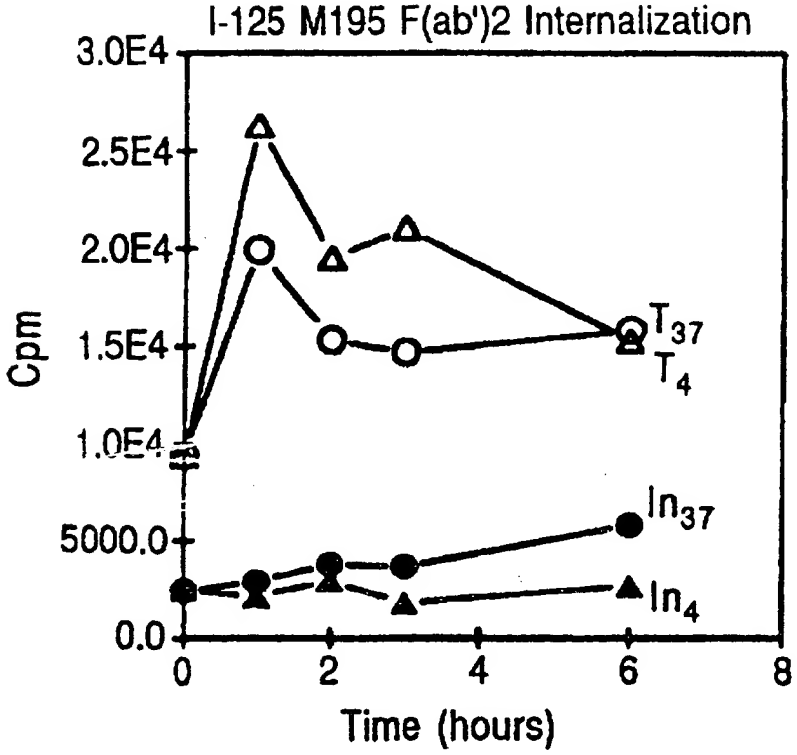


FIG. 10B

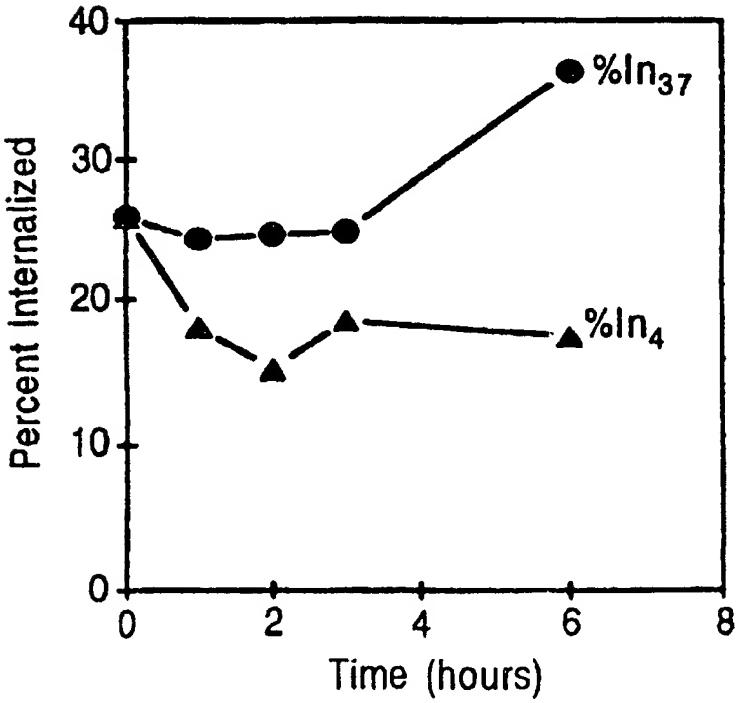


FIG. 10C

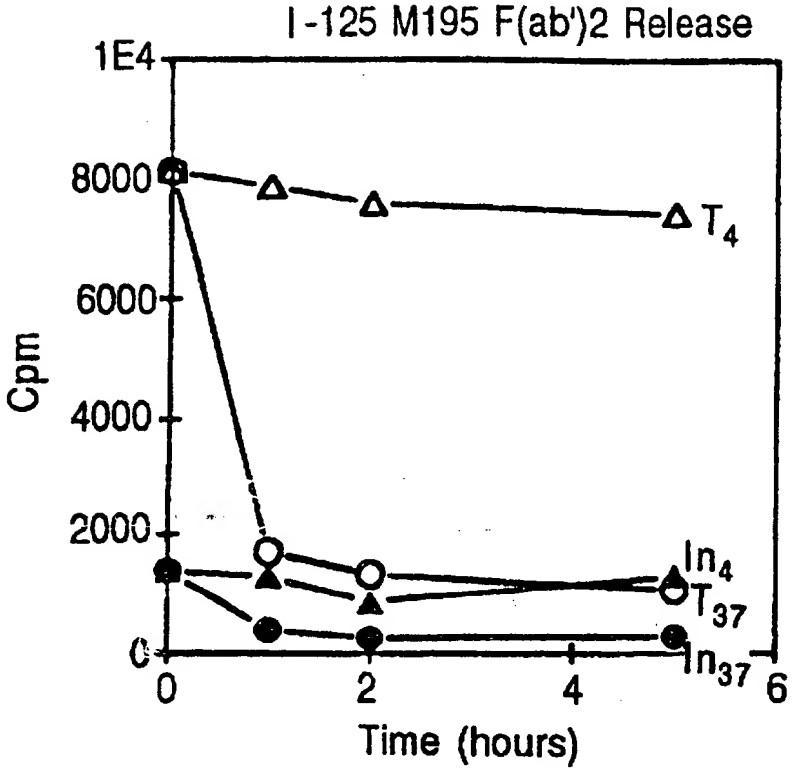


FIG. 10D

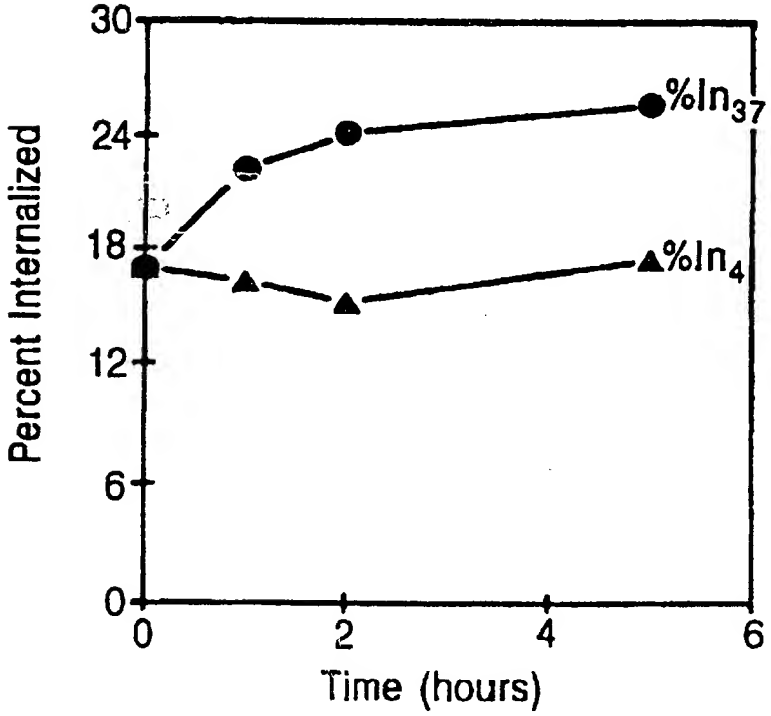


FIG. 11

Expression of The M195 Antigen

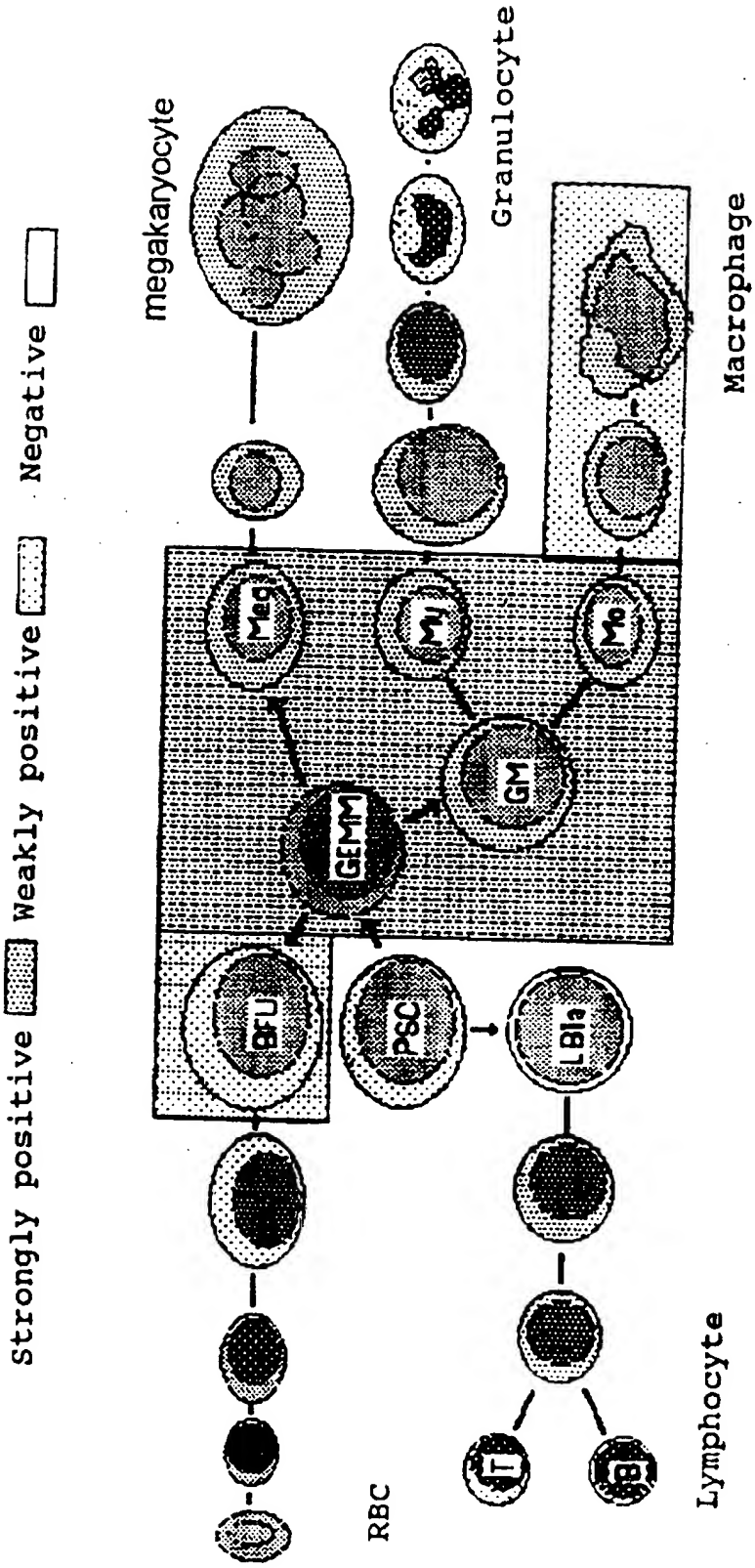


FIG. 12

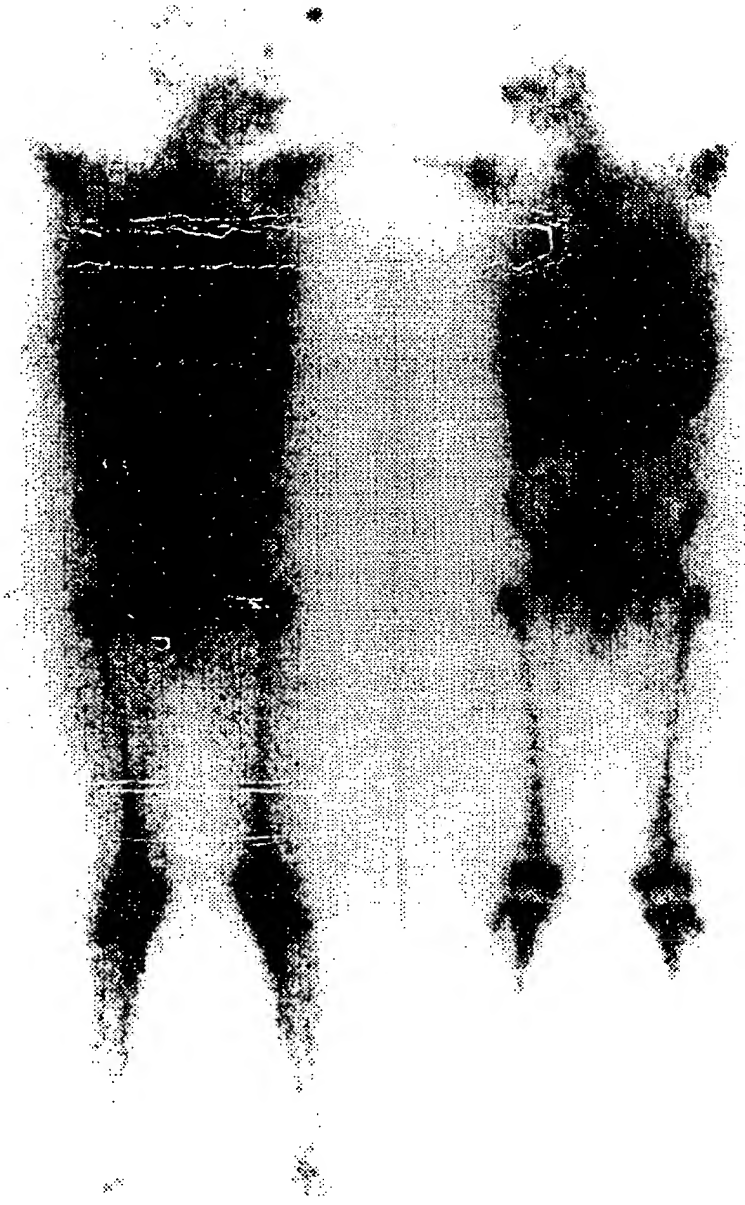
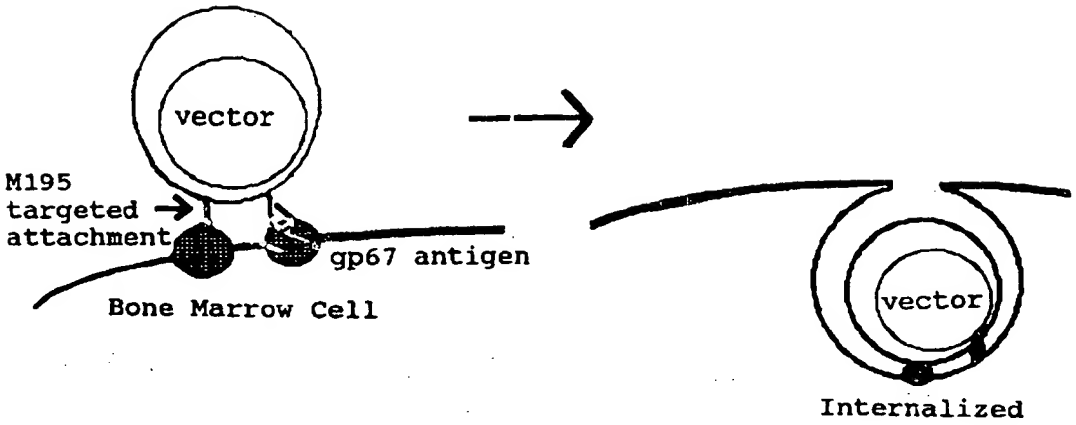
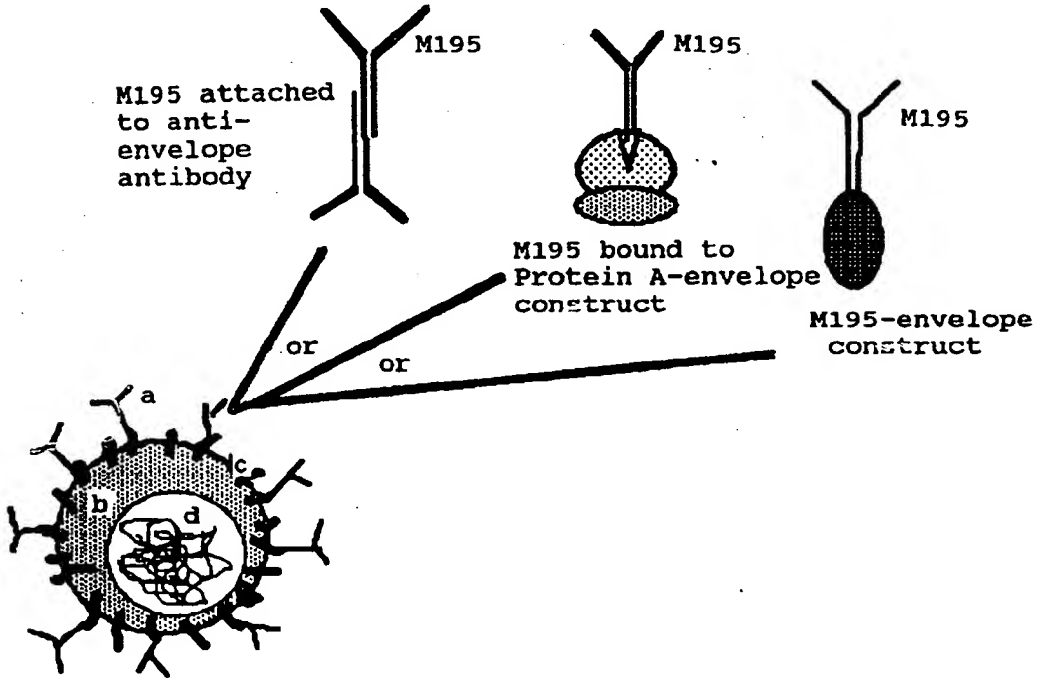


FIG. 13





a M195 or contruct
b retroviral vector
c envelope with protein and lipid
d genetic information

THERAPEUTIC USE OF HYPERVARIABLE REGION OF MONOCLONAL ANTIBODY M195 AND CONSTRUCTS THEREOF

This is a continuation of U.S. application Ser. No. 08/056,957, filed May 3, 1993, which is a continuation of U.S. Ser. No. 07/450,918, filed Dec. 14, 1989, now abandoned, the contents of which are hereby incorporated by reference.

The invention disclosed herein was made with Government support under NIH Grant No. CA08748 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various publications are referenced by numbers within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosure of these publications, in their entireties, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Mouse monoclonal antibody M195 is an IgG2a developed at Sloan-Kettering Institute (Tanimoto M., Scheinberg D A, Cordon-Cardo C, et al. Leukemia 3:339–348, 1989. Scheinberg D A, Tanimoto M, McKenzie S, et al. Leukemia 3:440–445, 1989.) which reacts with 60–70% of samples of blasts from patients with ANLL. M195 also binds to early myeloid cells (CFU-GM) and some monocytes but not to the earliest myeloid progenitors. The target antigen is not expressed on any other hematopoietic or non-hematopoietic tissue. Antibodies to a related antigen on the same protein (CD33), My9 and L4F3, are currently being used to purge bone marrow of ANLL before autologous transfusion (Bernstein I D, Singer J W, Andrews R G, et al. J Clin Invest 79:1153–1159, (1987); Griffin J D, Linch D, Sabbath K, et al. Leukemia Res 8:521–534, 1984.). M195 is rapidly internalized into cells after binding and this effect can enhance delivery of radiometals, radioiodine or conjugated toxins into cells (Divgi C R, Minniti J G, Old L J, Scheinberg D A Amer Assoc Cancer Res 30: Abs #1606, 1989). M195 is able to kill leukemia cells with rabbit or guinea pig complement, but not by use of human complement or human antibody dependent cellular cytotoxicity in vitro. Activation of these mediators in vitro has correlated with these effects in vivo (Houghton A N, Mintzer D, Cordon-Cardo C, et al. Proc Natl Acad Sci USA 82:1242–1246, (1985)), but it is not known if the lack of in vitro effects will predict lack of in vivo effects. Because M195 also reacts with early myeloid cells, normal marrow progenitors may be affected also.

Long-term survival in ANLL with the best current chemotherapeutic regimens is generally less than 20% (Clarkson B D, Gee T S, Mertelsmann R, et al. CRC critical review in Oncology/Hematology 4:221–248 (1986)). Survival of patients who relapse or who fail first attempts at induction chemotherapy is far lower. Autologous or allogeneic bone marrow transplantation may improve survival, but only in a small subset of patients (Gale R P, Horowitz M M, Biggs J C, et al. Lancet 8647:1119–1121 (1989)). There are no effective therapies for myelodysplastic syndromes or chronic monocytic leukemias and long term survival in these diseases is rare. Among patients with chronic myeloid leukemias (CML), only allogeneic bone marrow transplant has had an impact on survival (Clarkson B D J Clin Oncol 3:135–139 (1985)).

(Ref.a "Mab195: A Diagnostic Marker . . . "): Monoclonal antibodies (mAb) reactive with differentiation antigens present on myeloid cells and their progenitors are being used to study hematopoietic differentiation, to identify acute nonlymphoid leukemia (ANLL), to study the effects of hematopoietic growth factors, to purge bone marrow of leukemia cells, and for therapy in vivo (1–15 Ref.a).

(Ref.b "Restricted Antigens . . . "): The antigens displayed on the surface of acute non-lymphocytic leukemia (ANLL) cells and hematopoietic progenitor cells are being mapped in a number of laboratories using monoclonal antibodies (mAbs) (1 Ref.b). These studies have been directed at identifying antigens that are useful in distinguishing lymphoid from nonlymphoid leukemias (2–4 Ref.b), in subtyping of acute myelogenous leukemia, and in predicting outcome (5–10 Ref.b) and in therapy in vivo (11 Ref.b) or via bone marrow purging ex vivo (12 Ref.b). Antigens defining ANLL cells also identify normal hematopoietic cells during early stages of their development and thus should be classified as differentiation antigens rather than leukemia specific antigens.

Antigens restricted to the earliest stages of hematopoietic development are of particular interest since ANLL is thought to be derived from these cells (13–16 Ref.b). mAbs identifying these early cells can help in their purification or the study of growth regulation and control of differentiation (17 Ref.b). Such early progenitors may be useful for autologous reinfusion in bone marrow rescue (18 Ref.b). Studies of bone marrow from patients with ANLL have shown that the clonogenic cells are probably derived from a subset of cells which are phenotypically more immature that the majority of cells in circulation (14, 15 Ref.b). This suggests that analysis of the development of leukemia cells, as well as therapeutic trials, should also be directed at these early cells and not simply the phenotypically predominant cells in the marrow and peripheral blood.

Several mAbs restricted to hematopoietic progenitors have been described: monoclonal antibodies MY10, 3C5, and 12.8 recognize a 115-kDa glycoprotein (gp115 [CD34]) found on normal colony forming cells, myeloblasts, and leukemic blasts from most patients with ANLL and acute lymphoid leukemias (19–21 Ref.b). mAb NHL-30.5 identifies a 180-kDa protein found on a similar distribution of cells (22, 23 Ref.b). My9 and L4F3 antibodies identify a 67-kDa glycoprotein (CD33) (24–27 Ref.b) which is expressed on slightly more mature progenitors (subsets of CFU-GEMM and some older cells) and is restricted to leukemias of the myeloid and monocytic lineage. Long-term culture studies suggest that elimination of cells bearing the CD33 antigen will still allow regrowth of normal marrow cells of all lineages, presumably because of the presence of more immature antigen negative progenitors (25 Ref.b). Sabbath et al. (15 Ref.b) show that the CD33 antigen is expressed on leukemic colony-forming cells whereas other more mature markers are less commonly expressed. Finally, studies with ANLL marrow suggest it may be possible to purge leukemia cells from the bone marrow of many patients with ANLL using complement fixing antibodies to CD33 without destroying the ultimate normal progenitors (24 Ref.b). Several other antibodies with a less restricted distribution have also been described (14, 28, 29 Ref.b).

(Ref.c): Since the discovery of hybridoma technology by Kohler and Milstein (1Ref. c), there has been considerable interest in the utility of monoclonal antibodies as carriers of radioactivity for the diagnosis and therapy of cancer (2,3Ref. c). After the initial report by Goldenberg et al. on the utility of radiolabeled antibodies in the detection of cancer (4), there have been several clinical trials utilizing radiolabeled monoclonal antibodies in lymphoma and leukemia (5–11

Ref.c), both for radioimmunolocalization and radioimmunotherapy. Most of these trials have employed radioiodine (5–10 Ref.c); Carrasquillo and associates have also studied ^{111}In-labeled monoclonal antibody T101 (5.9 Ref.c) in the diagnosis of T-cell lymphoma. One obvious advantage of radiolabeled antibodies is that the specificity of antibody for the target antigen, often expressed in increased quantities on neoplastic cells, offers a potentially useful method for the selective delivery of radioactivity to the tumor site; moreover, the range of potentially lethal radiation emitted by most currently used radionuclides extends over several cell diameters, making it theoretically possible for the radiation to be cytotoxic to neighboring neoplastic cells that lack the target antigen.

Historically, beta-minus particle emitters such as ^{131}I have been preferred for mAb directed radioimmunotherapy. Radionuclides such as ^{125}I that decay by electron capture are also of interest in radioimmunotherapy because they are cytotoxic when internalized by the cell nucleus (12 Ref.c). ^{125}I labeled antibodies that are internalized into the cell following interaction with the target antigen may thus be cytotoxic (13 Ref.c). Studies in both animals and humans have shown that the radiometal ^{111}In concentrates to a significantly greater extent in tumor compared to radioiodine (14–17 Ref.c). Thus, use of beta-minus emitting radiometals such as ^{90}Y are of interest for therapy as well. Therefore, the choice of radionuclide used to label monoclonal antibodies may be of importance in the design of clinical trials utilizing radiolabeled mAbs for diagnosis and therapy.

Antigen-antibody complexes may either be shed from the cell or internalized into the cell following interaction with antibody. This process, known as modulation, was first described in mice (18 Ref.c) and later confirmed to occur during trials of mAb in humans (19 Ref.c). The process appears to be a general phenomenon found in many antigen-antibody systems of hematopoietic cells (20 Ref.c) and neoplasms as well as in solid tumors (21 Ref.c). Modulation may result in mAb shedding, internalization, or both processes. Shedding may result in residence time of the antibody on the target cell too short to achieve cell kill. On the other hand, internalized antigen-antibody complexes may theoretically deliver significant amounts of cytotoxic antibody into the cell if the cytotoxic label attached to the antibody is internalized into the cell and retained.

The cell biology of modulation and receptor internalization has been studied elsewhere (22,23 Ref.c).

SUMMARY OF THE INVENTION

This invention provides a recombinant polypeptide which comprises an amino acid sequence which is substantially the same as the amino acid sequence of the hypervariable region of monoclonal antibody M195 (ATCC No. HB 10306).

The invention further provides a chimeric antibody which comprises such a polypeptide, particularly in combination with the amino acid sequences of a human framework region and of a constant region from a human antibody.

Still further, this invention provides a therapeutic agent comprising such a chimeric antibody and a cytotoxic agent for example, a radioisotope or a toxin conjugated thereto. Also, this invention provides a therapeutic agent comprising monoclonal antibody M195 (ATCC No. HB 10306) and a cytotoxic agent conjugated thereto.

This invention additionally provides methods of treating or diagnosing acute or chronic leukemia in human patients, of effecting bone marrow transplants, and introducing genetic information into leukemia cells.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A–1C. Radiobinding assays of M195 IgG and F(Ab)'2: Saturation and Scatchard analysis. Assays were conducted as described in Materials and Methods. FIG. 1A, Total (◇), nonspecific (binding of ^{125}I-IgG in the presence of excess unlabeled IgG (♦), and specific (■) binding of M195 IgG to HL60 leukemia cells, FIG. 1B, Scatchard plot of M195 IgG binding. FIG. 1C, Scatchard plot of M195 F(Ab)'2 binding.

FIG. 2. Radioimmunoassay of M195 IgG and F(Ab)'2 on cell lines of hematopoietic origin. ^{125}I-M195 binding was determined at saturation as described in Materials and Methods. Nonspecific binding was 0.2 ng 500,000 cells. Only specific binding is shown: IgG, ■; F(Ab)'2, ■

FIG. 3. Complement cytotoxicity by M195 IgG on HL60 cells using rabbit complement. The concentration of rabbit complement is shown in the figure: final dilution of ⅙ (■), 1/18 (◇), and 1/30 (♦). The assay was conducted as described in Materials and Methods.

FIG. 4A and 4B. Indirect immunoperoxidase assay of M195 on trophoblast cells. FIG. 4A, photomicrograph of M195 IgG binding to trophoblast. FIG. 4B, Control IgG binding to trophoblast cells.

FIG. 5. Antigenic modulation after exposure of HL60 cells to mAb M195. The assay was conducted as described in Materials and Methods. M195 IgG was added at the concentrations shown in the figure and allowed to incubate at 37° C. for times indicated on the X axis. Cells were then tested for lysis by an additional aliquot of M195 IgG with rabbit complement at 37° C. for 45 min. Cytotoxicity after this second addition is shown on the y axis.

FIG. 6. Direct Radioimmunoassay for mAb M195 IgG on fresh hematopoietic neoplasms. The assay was conducted as described in Materials and Methods. The identities of the specimens are shown along the X axis; 1 ng bound per 500,000 cells is equivalent to 8000 IgG per cell. AMOL is acute monocytic leukemia; CML-MB is myeloblastic CML; MM is multiple myeloma; HCL is hairy cell leukemia; CLL is chronic lymphocytic leukemia; DPDL is diffuse poorly differentiated lymphoma; DHL is diffuse histiocytic lymphoma; NPDL is nodular poorly differentiated lymphoma; The lymphomas were suspensions made from lymph nodes. Background nonspecific binding was 0.2 ng bound. Only specific binding is shown.

FIG. 7. Blocking of M195 direct radioimmunoassay by excess unlabeled Ig. A 50–100 fold molar excess of the antibodies designated along the X axis were added to HL60 target cells followed by ^{125}I-M195 at 4° C. for 60 min. The amount of bound ^{125}I-M195 is shown on the Y axis. Binding of M195 without competing IG was normalized to 100%

FIG. 8A–8D. ^{125}I-M195 Internalization & Release.

Internalization (FIGS. 8A, 8B): 5 million HL60 cells were mixed with 5 μg mAb or fragment and incubated at 4° or 37°. Aliquots were taken at appropriate time points and measured for total and internalized cell-associated radioactivity.

All graphs showing radioactivity in counts per minute over time are labeled as follows: (Δ—Δ): total cell-associated radioactivity at 4° C.; (○—○): total cell-associated radioactivity at 37° C.; (▲—▲): internalized radioactivity at 4° C.; (●—●) internalized radioactivity at 37° C.

All graphs showing percent internalized radioactivity are labeled as follows (▲—▲): percent internalized at 4° C.; (○—○); percent internalized radioactivity at 37° C.

Release (FIGS. 8C, 8D): 5 million cells were incubated with 5 μg mAb or fragment at 4° for 60 minutes, then washed free of ambient mAb. Measurements of internalized radioactivity were carried out as described above with the washed cells being kept at 4° or 37°. The symbols for these graphs are as for internalization data.

FIGS. 9A–9D. ^{111}In-M195 Internalization & Release.

Internalization (FIGS. 9A, 9B): Conducted exactly as described in FIGS. 8A, 8B except M195 is now labeled with In-111.

Release (FIGS. 9A, 9B): Conducted exactly as described in FIGS. 8C, 8D except M195 is now labeled in this experiment with In-111.

FIGS. 10A–10D. ^{125}I-M195 F(ab')2 Internalization & Release.

Internalization (FIGS. 10A, 10B): Conducted exactly as described in FIGS. 8A, 8B except I-^{125} is now attached to $F(ab')_2$ fragment of M195.

Release (FIGS. 10C, 10D): Conducted as described in FIGS. 8*c*, 8*d* except M195 $F(ab')_2$ is now labeled with I-125.

FIG. 11. Schematic diagram of the distribution of the M195 antigen in human tissues. The antigen is not known to be present on any adult non-hematopoietic tissues, so they are not shown. The distribution in the hematopoietic cells is shown.

FIG. 12. Posterior and anterior whole body gamma camera images of patient #1, injected 18 hours earlier with 5 mCi iodine-131 M195 (1.5 mg). All known areas of leukemic involvement (bone marrow, spleen, liver, mediastinal chlorama) show marked uptake of M195.

FIG. 13. Schematic diagram of the method of antibody targeting of genetic information into hematopoietic cells

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a recombinant polypeptide which comprises an amino acid sequence which is substantially the same as the amino acid sequence of the hypervariable region of monoclonal antibody M195 (ATCC No. HB 10306).

The hybridoma which produces the monoclonal antibody designated M195 has been deposited with the American Type Culture Collection in Rockville, Md., U.S.A. 20852, under ATCC Accession No. HB 10306 on Dec. 14, 1989. This deposit was made pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (Budapest Treaty).

Also, one may obtain such a polypeptide by nonrecombinant methods, such as for example, proteolytic digestion.

A chimeric antibody comprising such a recombinant polypeptide is also provided, particularly a chimetic antibody comprising the amino acid sequences of a human framework region and of a constant region from a human antibody so as to "humanize" or render nonimmunogenic the hypervariable region of the mouse M195 monoclonal antibody.

This invention also concerns a therapeutic agent comprising such a chimeric antibody and a cytotoxic agent conjugated thereto.

Of particular interest are therapeutic agents wherein the cytotoxic agent is a radioisotope, such as an alpha particle emitter, for example one selected from the group consisting of Lead-212, Bismuth-212, and Astatine-212

In one embodiment of the therapeutic agent, the alpha particle emitter is conjugated to the chimeric antibody by means of a bifunctional chelate.

Alternatively, the cytotoxic agent present in the therapeutic agent may be a beta particle emitter, e.g. one selected from the group consisting of Iodine-131, Scandium-47, Rhenium-186, Rhenium-188, and Yttrium-90.

Still further, the radioisotope may be an auger electron generator, e.g. one selected from the group consisting of Iodine-123, Iodine-125, Bromine-77, and Indium-111, or a fissionable nuclide such as Boron-10 or an Actinide.

A therapeutic agent comprising monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto is also provided by this invention, e.g. one in which the cytotoxic agent is a radioisotope.

Also, this invention concerns a polypeptide comprising the recombinant polypeptide described hereinabove fused to another polypeptide, for example, a toxin or a drug.

This invention also provides a method of treating acute or chronic leukemia in a human patient which comprises administering to the patient an amount of the therapeutic agent comprising a chimeric antibody to which a cytotoxic agent is conjugated, sufficient to bind to, and be internalized by, leukemic cells so as to thereby destroy the leukemic cells.

Further, the invention concerns a method of treating acute or chronic leukemia in a human patient which comprises administering to the patient an amount of a therapeutic agent comprising M195 conjugated to a radioisotope sufficient to bind to, and be internalized by leukemic cells so as to thereby destroy the leukemic cells.

In such methods the amount of therapeutic agent is typically from about 0.05 mg. to about 100 mg and the therapeutic agent is administered intravenously.

Still further, this invention provides a method of treating acute or chronic leukemia in a human patient which comprises administering to the patient an amount of a therapeutic agent as described hereinabove sufficient to bind to, and be internalized by, leukemic cells so as to thereby destroy the leukemic cells.

In one embodiment, the therapeutic agent comprises Iodine-131 and the sufficient amount comprises from about 50 mCi to about 200 mCi. In another embodiment, the therapeutic agent comprises Yttrium -90 and the sufficient amount comprises from about 10 mCi to about 50 mCi. In yet another embodiment, the therapeutic agent comprises Bismut-212 and the sufficeint amount comprises from about 20 mCi to about 80 mCi. In still another embodiment, the therapeutic agent comprises Iodine-123 and the sufficient amount comprises from about 100 mCi to about 300 mCi.

This invention additionally provides a method destroying a human patient's natural bone marrow cells which comprises administering to the patient an amount of the therapeutic agent according to the invention sufficient to destroy the patient's bone marrow cells under conditions such that the therapeutic agent binds to, is internalized by, and destroys the bone marrow cells.

Thus, the therapeutic agent may comprise monoclonal antibody M195 (ATCC Accession No. HB 10306) and a cytotoxic agent conjugated thereto, the amount of antibody may be from about 0.01 mg. to about 50 mg; and the therapeutic agent may be administered intravenously.

Additionally, this invention concerns a method of treating leukemia which comprises removing bone marrow cells, including leukemia cells, from a human leukemic patient; contacting the bone marrow cells so removed with a sufficient amount of the therapeutic agent of the invention to bind to, be internalized by, and thereby destroy the leukemia cells

present in the bone marrow cells; and autologously reinfusing the resulting bone marrow cells into the patient. Preferably, the contacting of the bone marrow cells, including leukemia cells, removed from the patient is effected in the presence of rabbit or guinea pig complement.

Additionally, this invention concerns a method of diagnosing acute or chronic leukemia in a human patient which comprises administering to the patient an antibody according to this invention labelled with an imaging agent under conditions so as to form a complex between the antibody and any leukemia cells present in the patient, imaging any complex so formed, and thereby diagnosing acute or chronic leukemia Preferably, the imaging agent is internalized into the leukemia cells.

In one embodiment, the antibody is monoclonal antibody M195 (ATCC Accession No. HB 10306) and the imaging agent is a radioisotope such as a positron-emitting radiometal; a gamma-emmitting radiometal; Iodine-131; Iodine-123; Indium-111 or Technetium-99m.

Finally, this invention provides a method of introducing or carrying genetic information into leukemia cells which comprises contacting cells with the antibody of this invention to which the genetic information is attached or with which it is associated, so that the antibody binds to the cells to form a complex, which is thereafter internalized into the cells, so as to thereby introduce or carry the genetic information into the cells.

In one embodiment, the genetic information is in a retroviral vector attached to the antibody.

EXPERIMENT 1

This experiment and in experiment 2 describes a mouse monoclonal antibody, M195, which defines an antigen restricted to early myeloid cells, monocytic cells, and ANLL. The antigen appears to be carried on the CD33 protein. The antigen is not detectable on any other adult tissues and thus may be useful in the study of myelomonocytic differentiation and in the diagnosis and therapy of ANLL. This experiment describes the distribution of the antigen on cell lines, normal tissues, and mature hematopoietic cells. The antibody's biological activity, affinity, and quantitative distribution on individual cells are presented.

MATERIALS AND METHODS

mAbs. mAb M195 was produced from hybridomas resulting from a fusion of SP2/0-Ag14 mouse myeloma cells and the spleen cells of a 5-week-old BALB/c mouse immunized with leukemia cells from a patient with ANLL (FAB-M2). Supernatant fluids from cloned hybridoma cultures were screened against a panel of leukemia cell lines and the original ANLL leukemia cells using *Staphylococcus aureus* protein A (PA) erythrocyte rosetting (see below). The repeatedly sub-cloned M195 hybridoma was expanded in the peritoneal cavity of doubly pristane-primed (C57BL/6× BALB/c) F1 mice.

M195 was purified on PA-Sepharose (Pharmacia) by affinity chromatography using sequential pH step elutions. Purity was determined on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie brilliant blue.

Control antibodies included mAb AJ2, reactive with a broadly expressed cell surface antigen (VLA) produced at Sloan-Kettering (31 Ref.b), and mAb M31 (reactive with the Lewis X antigen) developed in this laboratory (unpublished).

Screening of Hybridoma Supernatants. Four thousand cells (HL60) or the original immunizing ANLL cells) in 10 µl of RPMI with 10% fetal calf serum (FCS) were allowed to settle and attach to concanavalin A-coated (Pharmacia) Terasaki 60-well plates (NUNC) for 45 min at 20° C. as described (32, 33 Ref.b). Hybridoma supernatants were tested for reactivity on these cells using PA-coated human O^- red blood cell rosettes as indicators (32 Ref.b).

Cells and Cell Lines. Heparinized peripheral blood samples and bone marrow aspirates were obtained from healthy volunteers and patients on the Leukemia Service at Memorial Hospital after informed consent. Mononuclear cells were separated on Ficoll-Paque (Pharmacia), and adherent cells were isolated from the nonadherent mononuclear cells by plastic adherence for 2 hr. at 37° C. Polymorphonuclear leukocytes were purified from contaminative red blood cells after dextran sedimentation at 1×g for 60 min by ammonium chloride lysis in Tris buffer at pH 7.2. Platelets were separated from the Ficoll-Paque interface cells by differential centrifugation. E-rosette-positive and negative fractions of mononuclear cells were separated after incubation and neuraminidase- (Calbiochem) treated sheep red blood cells (GIBCO), followed by Ficoll-Paque gradient centrifugation and lysis of red cells with ammonium chloride.

Hematopoietic cell lines (Table 1) and nonhematopoietic cell lines (Table 3) were obtained from the human tumor banks of the Human Cancer Immunology Laboratory at Sloan-Kettering Institute. 1F10 and 1F10 (mono), an HL60 subclone and its monocytic differentiated form, were the gift of Dr. Y. Cayre, Sloan-Kettering Institute (34 Ref.b).

Serologic Assays: Immune Rosetting. Antibody specificity was determined on adherent cell lines plated in 60 well Terasaki plates using Staphylococcus aureus PA or rabbit antimouse Ig-coated human O red blood cells prepared as described (32 Ref.b) as indicators. Suspension target cells are assayed using the same indicator cells except that the target cells were attached to Terasaki plates immediately before testing using Concanavalin A (33 Ref.b). This assay is sensitive to concentrations of mAb M195 of about 1 ng/ml binding to HL60. Cells were considered negative if no rosettes formed below an ascites dilution of 200 and absorption analyses were also negative. Ascites fluids were considered "weakly positive" on a cell line if greater than 50% of cells formed rosettes at dilutions between 200 and 100,000. "Weakly positive" cells were confirmed as reactive by absorption analysis (see below). Ascites from mice bearing hybridomas were considered positive with a cell line if rosetting of cells occurred at a dilution of greater than 100,000. If purified antibody was used, cell lines were scored "positive" for rosetting at concentrations below 50 ng/ml and "weakly positive" at concentrations of 50–500 ng/ml. Reactivity was also confirmed by direct radioimmunoassay and by complement fixation assays (see below).

Absorption Analysis. Two to ten million cells were washed in PBS and pelleted at 500×g in a 5×50 mm glass tube and allowed to react with an equal volume of ascites diluted to a concentration four times that needed to form 50% rosettes on positive cells: HL60 cells or the immunizing ANLL cells. (This was typically a dilution of ascites of 100,000–200,000.) The absorption proceeded for 30 min. at 4° C., and the mixture was again pelleted at 500×g. The supernatant was reacted with target cells lines in rosetting assays as described above.

Antibody-dependent Cellular Cytotoxicity (ADCC). Assays to determine if M195 was capable of mediating ADCC were conducted essentially as described by Welt et al. (35 Ref.b). Target cells were incubated in ^{51}Cr for ninety

minutes and then washed of free ^{51}Cr. M195 antibody was added at concentrations of 1–100 μg/ml on ice, and fresh peripheral blood mononuclear cells added at effector to target ratios of 10–40/1. Cells were incubated at 37° C. for 6–18 hr and harvested using a Skatron cell harvester, and released ^{51}Cr was counted in a Packard gamma counter. Detergent lysed cells were used as a 100% control, and isotype matched irrelevant antibody treated cells were used as a negative control.

Radioiodination and Radioimmunoassays. Purified antibodies were labeled with Na-^{125}I (New England Nuclear) using chloramine-T to start and sodium metabisulfite to stop the reaction. Specific activity was between 2 and 10 μCi/μg of protein. Immunoreactivity was between 40 and 60% as determined by serial binding to an excess of live HL60 cells. Radioimmunoassays were conducted on 5×10^6 live cells in 100 μl RPMI with 10% FCS and preincubated 15 min. with 2% heat inactivated normal rabbit serum to block nonspecific binding. Binding proceeded at 4° C. for 90 min followed by the tree washes with RPMI/FCS. Bound radioactivity was measured in the cell pellets in a Packard gamma counter.

Preparation of F(ab)'2 Fragments. One mg of purified immunoglobulin was reacted at 37° C. for 6 hr with immobilized pepsin beads (Pierce Chemicals) in acetate buffer at pH 4.5. The reaction was stopped by adjusting the pH to 8.8 and sedimenting the pepsin beads at 15,000×g for 1 min. Undigested immunoglobulins and Fc fragments were removed by reaction with Protein A Sepharose (Pharmacia). Purity of fragments was determined by SDS-polyacrylamide gel fractionation followed by Coomassie blue staining.

Competition Radioimmunoassay for Blocking Antigen in Serum. Serum from patients with hematopoietic neoplasms was obtained from fresh clotted blood and stored at −70° C. until use. The presence of blocking M195 antigen in sera was assayed by incubating 50 μl of freshly thawed serum and a dilution of ^{125}I-labeled mAb M195 IgG for 20 min at 4° C. M195 IgG was at a concentration sufficient for 50% maximal binding to 5×10^5 HL60 target cells. The cells were then added and the incubation continued for 60 min at 4° C. followed by two washes with RPMI medium. Inhibition of M195 IgG binding was scored as the percent decrease in binding to HL60 as compared to mAb M195 incubated in the presence of 2% bovine serum albumin (BSA) in PBS and no competing sera.

Complement-mediated Cytotoxicity. Twenty-five μl of target cells at 2×10^6 cells/ml were mixed with 25 μl of complement and 25 μl monoclonal antibody at 4° C. The mixture was then incubated at 37° C. and occasional shaking for 45 min. Live and dead cells were enumerated using trypan blue exclusion as an indicator.

Guinea pig serum and baby rabbit serum were purchased from PelFreeze; human sera were obtained from volunteers. All complement sources were stored at −70° C. until use and not reused. Complement was used at the maximum concentrations not showing nonspecific lysis of the target cells: generally at 1:6–8 final dilution.

Indirect Immunoperoxidase and Immunofluorescence Assays. Histologically normal adult human tissues were obtained from surgical pathology specimens within 1–2 hr of resection. Several normal specimens of organs from several cases were used. Tissues were embedded in OCT compound after freezing in iopenthane/liquid N_2. Tissues were cut 4–8 μm thick, fixed in acetone, quenched with 0.1% H_2O_2, and blocked with either goat or horse sera. MAb M195 was used as supernatant, ascites, or purified IgG at 20 μg/ml. Positive and negative Ig controls were included in all studies. Goat anti-mouse IgG peroxidase conjugates (1:50 dilution) (Tago, Burlingame, Calif.) or biotinylated horse anti-mouse IgG with Avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, Calif.) were used as secondary reagents. Diaminobenzidine was used as a chromogen. For fluorescence studies, goat anti-mouse Ig fluorescein isothiocyanate conjugates (Becton-Dickenson) were used as secondary reagents.

Modulation of Cell Surface Antigen. Modulation of the cell surface antigen detected by mAb M195 after antibody binding was monitored by complement mediated cytotoxicity (36 Ref.b). HL60 cells were incubated with various concentrations of M195 IgG for up to 3 hr at 37° C. Additional antibody and rabbit complement were added at several time points and the amount of cell lysis was determined 45 min later.

Differentiation of HL60. A cloned variant of HL60, IF10 (34 Ref.b), and its differentiated monocytic variant (incubation with vitamin D_3 and phorbal myristate acetate for 3 days to promote monocytic maturation) were used (34 Ref.b). Both cell lines were kindly provided by Dr. Y. Cayre.

RESULTS

Distribution of M195 Antigen on Hematopoietic Cell Lines. mAb M195 was selected for detailed study from a group of several hundred hybridoma-produced antibodies generated from the fusion of a spleen from a mouse immunized with fresh live ANLL cells (FAB classification, M2). The antibody showed specific high titer binding in PA-rosette assays to the myeloid and monocytic cell lines. HL60, KG1, IF10, U937, and the monocytic variant of IF10 (Table 1). mAb m195 was weakly reactive with the erythroleukemic line K562 and not reactive with KG1a, an undifferentiated myeloid line. mAb M195 did not react with 18 lines of B cell origin at various stages of differentiation nor with 10 lines of T cell derivation. One null lymphocytic line, N-ALL-1, was weakly reactive. Activated B cells and activated T cells did not express antigen. Non-reactive cell lines were confirmed as negative by absorption assays which can detect about 1 ng of M195 binding in 1,000,000 cells; rosetting assays detect binding at antibody concentrations of about 1 ng/ml. mAb AJ2 was used as a positive control in these assays where most cells were M195 antigen negative. This panel of cell lines showed that among hematopoietic cells M195 was restricted to the nonlymphoid lineages: it was most highly expressed on committed myeloid and monocytic cell lines and more weakly expressed on erythroid and earliest myeloid cells.

TABLE 1

Reactivity of M195 with Hematopoietic Cell Lines

Cell	M195	AJ2 (positive control)
Myeloid	WP	P
K562, HL60	P0	PP
KG-1, KG1a	P	P
IF10		
Monocytic		
U937, 1f10	PP	PP
THP-1	0	

TABLE 1-continued

Reactivity of M195 with Hematopoietic Cell Lines

Cell	M195	AJ2 (positive control)
Pre-B cells		
NALL-1, NALM-1	00	P
NALM-6, NALM-16	00	
B cells		
SK Ly-16, -18, Daudi,	000	P
ARA-10, SK DHL-2, Raji	000	PP
CCRF-SB, LICR/My-2	00	P
BALL-1		
Myelomas		
Oda, U266, RPMI 8266	000	P
RCS, HAS, Brown	000	
EBV-transformed B cells (n = 15)	0	
T cells		
T-45, CCRF-CEM, Molt 4	000	P
TALL-1; MT-1, HUT-102	000	
RPMI 8402, CCRF-HSB2	00	
p12/ichikawa, HPB-ALL	00	P
PHA blasts (n = 5)	0	

P = positive; W = weakly positive; 0 = negative
*As determined by direct Protein A and mixed heme-adherence rosetting and absorption assays as described in the text.

Quantitative Analysis of Binding to Myeloid Cell Lines. In order to confirm the results of rosetting assays and absorption assays and to look at quantitative differences in the expression of the M195 antigen among the myeloid and monocytic cells a sensitive radioimmunoassay using direct binding of ^{125}I-labeled purified M195 was used. Many of the hematopoietic cells have Fc receptors in addition to or instead of target antigen on their surfaces, and binding of radiolabeled IgG to these Fc receptors may confound the quantitative results of the radioimmunoassay. Therefore an F(ab')2 fragment of M195 was prepared and used in the assays to confirm the number of antigenic sites.

Binding of M195 IgG to HL60 showed saturation and specificity (FIG. 1A). Scatchard analysis showed an avidity of binding of 3×10^9 liters/mol (FIG. 1B) for the IgG. The number of binding sites calculated from this curve was approximately 100,000 per live HL60 cell. Scatchard analysis of several lots of M195 IgG on different passages of HL60 gave equivalent results. Analysis of purified F(ab)'2 of M195 (FIG. 1C) showed similar avidity (10^9 liters/mol) cells and numbers of binding sites (10,000/HL60 cell), suggesting that binding activity was not significantly altered by protease digestion of the fragment.

Both the intact IgG and the F(ab)'2, fragment were used for radioimmunoassays on hematopoietic cell lines (FIG. 2). Non-specific binding (binding of ^{125}I-M195 in the presence of excess unlabeled M195) under the conditions of this assay was approximately 200 pg (1600 molecules) per 5×10^5 cells. Therefore, only binding above this level was considered significant. Since the assay was done under saturating concentrations of M195 IgG or F(ab)'2, the total binding could be used to calculate the number of sites per cell. HL60, IF10, and U937 had 6000–12,000 sites per cell. KG1 had about 3000 sites per cell. Binding to KG1a and K562 was not above the background of nonspecific binding (1600 sites), and the nonmyeloid cell lines were negative. The assays confirmed the specificity of M195 for these myeloid and monocytic cells and showed that binding was not Fc receptor related. The three cell lines positive by rosetting and absorption had similar quantities of M195 antigen expression.

Reactivity with Fresh Normal Hematopoietic Cells. M195 was tested by absorption analysis for reactivity with live peripheral blood elements and cells derived from the major hematopoietic organs (Table 2). mAb M31 was used as a positive control. No reactivity was seen with M195 on any of these cell types.

Quantitative Analysis of Binding to Hematopoietic Cells. Direct radioimmunoassays were performed on fresh hematopoietic cells to confirm reactivity and quantitative binding (Table 2). Red blood cells, platelets, spleen cells, bone marrow cells, and peripheral blood mononuclear cells were negative. Polymorphonuclear leukocytes showed binding to the intact IgG at about 800 sites per cell above background but did not show significant binding to the F(Ab)'2 fragment suggesting that even this minimal binding was via the Fc receptors. Peripheral blood adherent cells (macrophages) were positive and binding to the F(Ab)'2 showed about 5000 antigen sites per cell. Binding to peripheral blood E-rosette negative cells was marginally above background, possibly due to the presence of a small percentage of macrophages contained in this population. With the exception of macrophages, the direct radioimmunoassays shown here confirmed the specificity analysis by absorption. The lack of reactivity with macrophages in the absorption assay may be due to the inability to obtain the large quantity of viable cells containing enough antigen necessary to absorb M195 (1,000,000 macrophages with 5000 antigen sites per cell would absorb only about 1 ng of antibody). Lack of binding in these radioimmunoassays would rule out the presence of some M195 positive cells within a large heterogeneous population as in bone marrow, for example.

TABLE 2

Cell Type	Assay Type: Absorption	Radio-immunoassay	Complement Lysis
T-enriched PBL[b]	—	—	—
B-enriched PBL	–	–	–
Granulocytes	–	–	–
Adherent monocytes	–	–	+
Platelets	–	–	ND[c]
Red blood cells	–	–	ND
Nonadherent PBMC	ND	–	–
Splenic T enriched	ND	–	–
Splenic B enriched	ND	–	–
Splenic mononuclear	–	ND	–[d]
Bone marrow mononuclear	–	–	–[d]
Lymph node mononuclear	–	ND	ND
Fetal thymocytes	–	ND	ND

[a]Conducted as described in the text
[b]PBL = peripheral blood lymphocytes; PBMC = peripheral blood mononuclear cells.
[c]ND = not done
[d]Nonadherent cells

Complement-mediated Cytotoxicity Assays. Complement-mediated cytotoxicity was also used to confirm specificity. Assays were first done to determine if mAb M195 was capable of killing cells in the presence of rabbit, guinea pig, and human sera as sources of complement. Enzyme-linked immunosorbent assays showed mAb M195 to be an IgG2a class immunoglobulin, which is generally able to fix complement. Using HL60 as targets, M195 was

capable of killing cells in the presence of guinea pig and rabbit complement but not human complement. In the presence of human complement, killing rarely occurred and was only 10–15% above background at its highest. Cell lines not expressing the antigen were not killed. No killing occurred in the absence of antibody or a source of complement.

Cytotoxicity was antibody concentration dependent and complement concentration dependent (FIG. 3). However, at concentrations of 10 μg/ml or greater of M195, nearly all cells were killed even with rabbit serum diluted 30-fold.

The complement assay was used to confirm the specificity analysis derived from the absorption assays and radioimmunoassays (Table 2). Complement assays are not confounded by Fc receptor binding and are able to determine percentages of cells within a large population which are antigen positive. Assays were conducted at 10 and 100 μg/ml M195 with rabbit serum diluted to 1:18 final concentration. HL60 and fresh monocytic leukemias were used as positive controls and B cell lineage RAJI cells and chronic lymphocytic leukemia cells were used as negative controls. Complement and antibody alone controls were also included. Background killing was between 1–55% in the controls without antibody or complement and 5–10% in the spleen E-rosette negative cells. Because of this background of several percent, it is not possible to determine if positive cells are present in a sample at this level or lower.

Only one population of mature normal hematopoietic cells showed killing above background using M195 and rabbit complement: peripheral blood adherent cells. Among three samples of adherent cells, 35–50% of cells were killed, showing that a subpopulation of these cells expressed the M195 antigen. This assay confirmed the radioimmunoassay data.

In chronic myelogenous leukemia (CML) mononuclear cells, a low percent of cells (5–6%) were killed above background (not shown). The cells comprising the CML mononuclear cell population include blasts through band forms with a predominance of the more mature myeloid cells. Morphologic analysis of these cells before and after antibody and complement treatment did not show which cells, if any, had been selectively killed. Because peripheral blood cells from patients with CML represent the full spectrum of maturing myelogenous cells, this lack of significant cytotoxicity confirms the lack of reactivity of M195 with the vast majority of adult myelogenous cells.

Reactivity of M195 and Differentiated HL60 Cells. IF10 cells and differentiated monocytic IF10 cells were provided by Dr. Yvon Cayre. One hundred percent of the IF10 cells became morphologically changed and adherent. The reactivity of M195 was tested by both rosetting and radioimmunoassays before and after differentiation. In the differentiated monocytic 1F10 cells there was a 40% loss of antigen expression by radioimmunoassay. Rosetting assays remained positive but the titer of binding dropped 10-fold. Quantitative binding to the differentiated 1F10 was similar to fresh normal adherent monocytes, suggesting the loss of antigen with monocytic differentiation among fresh hematopoietic cells was paralleled by this model line in vitro.

ADCC Assays. M195 did not show any ability to mediate ADCC against HL60 cells or U937 under the conditions described in Materials and Methods. These cells are the highest expressors of the antigen among those tested.

Reactivity of M195 with Nonhematopoetic Cell Lines. M195 was tested for reactivity with 70 cell lines derived from a wide spectrum of cancers (Table 3). No reactivity was seen. Monoclonal antibody AJ2 was included as a positive control and was positive in every case tested. Therefore, the M195 antigen appears to be restricted to hematopoietic cells.

TABLE 3[a]

Reactivity of M195 with Non-Hematopoietic Cell Lines

		M195	AJ2 (positive control)
Astrocytomas	SK-MG-1,-2,-3.	000	P
	-4,-6,-7,-9	000	P
	-12,-15,17,-23	0000	P P
Bladder cancers	T-24, 253J,5637	000	
Breast cancers	SK-BR-3,-5,-7	00	
	BT-20,MCF-7	00	P
Cervical cancers	CC-A, CC-B, HT-3	000	PPP
	C41	0	P
Choriocarcinomas	GCC-SV(c), Lu-75(c)	00	
Colon cancers	SW-403,-480,-620	000	PPP
	-116,-1417	00	PP
	HT-29,SK-CO-10	00	PP
	CaCo-2,HCT-15	00	PP
Lung Cancers	SK-LC-1,-4-6	000	P P
	-8,9,-10,-17	0000	P P
	Calu-1,-6,Sz-Lu-1	000	PPP
	SK MES-1	0	P
Melomas	SK MEL-13,23,-28	000	PPP
	-29,-37,-93	000	PPP
	-173, MeWo	00	P
Neuroblastomas	SK-N-MC,PNDW	00	PP
Ovarian cancers	SK-OV-3,OV-2774	00	P
Pancreatic	ASPC-1,-2	00	PP
Renal cancers	SK-RC,-1,-2,-7	000	P
	-8,-9,-20,-28,	0000	PP
	-29,-45,-48	000	PPP
Uterine cancer	MB 180, SK UT-1	00	PP

[a]Conducted as described in the text

Tissue Distribution of M195. The reactivity of M195 with human tissues was determined in indirect immunofluorescence and indirect immunoperoxidase assays on fresh frozen tissue (Table 4). Among 25 different tissue types, reactivity was seen only with trophoblast. This reactivity was predominantly cytoplasmic (FIGS. 4A and 4B). These data on fresh tissue are consistent with the specificity data obtained from the assays with cell lines above.

TABLE 4

Tissue Distribution of M195[a]

Tissue	Fluorescence	Peroxidase
Adrenal	0	0
Bladder	0	0
Blood vessels	0	0
Brain	0	0
Breast	0	0
Capillaries	0	0
Cervix	0	0
Colon	0	0
Heart	0	0
Kidney	0	0
Liver	0	0
Lung	0	0
Lymph node	0	0
Ovary	0	0
Pancreas		0
Placenta	0	0
Prostate	0	0
Skin	0	0
Small Intestine	0	0
Stomach	0	0
Testis	0	0
Thyroid	0	0
Trophoblast		P

TABLE 4-continued

Tissue Distribution of M195*

Tissue	Fluorescence	Peroxidase
Ureter	0	0
Uterus	0	0
HL60 (positive control)	P	P

0 = negative; P = positive staining
*Conducted as described in the Materials and Methods

M195 Reactivity with Fresh Leukemias. M195 reacted with most myelogenous leukemias and rarely with lymphoid leukemias in rosetting assays. Because of the nature of the rosetting assay, it was not possible to determine which cells were reactive or what percentage of blasts were positive. These issues and a detailed analysis of the specificity and activity of M195 in comparison to standard cell surface markers are presented in the accompanying paper (30 Ref.b).

M195 Blocking Antigen in Serum. In order to determine if the M195 antigen was shed into sera from hematopoietic cells, sera from people with a variety of leukemias and lymphomas or from healthy individuals were tested for soluble antigen capable of blocking of the binding of radiolabeled mAb M195 to HL60 cells (Table 5). Three of 39 human sera blocked binding significantly. The blocking was not complete. One serum was from a patient with CML. Two sera of six patients with acute lymphocytic leukemia partially block binding. The leukemia cells from both of these patients were not reactive with M195 antibody suggesting that the blocking antigen was not shed from these cells or that the blocking activity was not specific. These data suggest that M195 antigen in the serum would not be capable of preventing mAb M195 from reaching target cells. Because the sensitivity of this assay is about 200 ng/ml of M195, it is possible that M195 is expressed at lower levels than this in sera. In addition, monovalent antigen with low avidity for the M195 IgG may be present but unable to block binding.

TABLE 5

M195 Blocking Factors in Sera of Patients with Leukemia

Serum Source	Number Tested	Number Blocking*
Normal	6	0
AML	13	0
CML	6	1 (52%)[b]
ALL	6	2 (56%,67%)
NHL/CLL	8	0
Rabbit, mouse, horse	5	0

*A serum able to reduce by 50% or more, direct binding of 200 ng/ml radioiodinated M195 to HL60 target cells.
[b]The percent reduction by each positive serum.

Antigenic Modulation. The ability of M195 to induce modulation of the antigen from the surface of HL60 cells was studied using complement-mediated cytotoxicity. HL60 cells were reacted with M195 at various concentrations, and the ability of M195 to kill the cells with added rabbit complement was measured versus time (FIG. 5). At the highest antibody concentrations complete modulation occurred within 3 hr. That is, the addition of complement to cells preincubated with mAb M195 for 3 hr resulted in no killing. Modulation was incomplete in cells exposed to lower mAb IgG concentrations. Other studies (to be published elsewhere) demonstrated that the modulation occurred via antigen internalization after antibody binding.

Biochemical Nature of the M195 Antigen. Treatment of HL60 cells with 100° C. for 1 min eliminated all binding activity in radioimmunoassays and rosetting assays. This suggested that the antigen epitope is carried on a protein. However, treatment with trypsin, protease, and neuraminidase had no effects on binding of mAB M195 to HL60 cells. These experiments, therefore, did not confirm the biochemical nature of the antigen. Repeated attempts to immunoprecipitate the antigen for ^{35}S-methionine-labeled cells or cells surface-labeled with iodine-125 using lactoperoxidase were unsuccessful. Wester immunoblotting on HL60 extracts were also negative. Although we were unable to identify the target, other data shown in the accompanying paper (30 Ref.b) indicated that the antigen was carried on the CD33 protein.

DISCUSSION

This paper details the specificity of a new mouse mAb, M195, which is reactive with myelogenous leukemias, early myeloid cells and some monocytic cells. Qualitative and quantitative analyses of the mAb's binding, its biological activity, and its immunological functions are described.

Since two potential uses of M195 are diagnosis and in vivo therapy of ANLL, a comprehensive definition of its reactivity with all tissues and cells of the body was undertaken. Several assays were used in the specificity analysis of M195 on fresh cells and cell lines. Rosetting assays which are sensitive enough to detect 1 ng of mAb M195 per ml were used initially for specificity analysis. Direct radioimmunoassays using iodine-125 IgG and F(Ab)'2 were used next in order to quantitate the number of antigen sites expressed on various positive cells. The F(Ab)'2 has the advantage of defining non-Fc receptor binding quantitatively. Finally, a complement fixation assay was used to analyze reactivity. Since biological activity after binding to antigen in an appropriate fashion is required in this assay, the effects of nonspecific binding are reduced. Indirect immunofluorescence followed by confirmation with indirect immunoperoxidase assays were used to define M195 antigen expression on a broad spectrum of normal tissues. These results supported data obtained from the rosetting and absorption analysis on cell lines. Because the tissues were frozen sectioned and fixed, binding to cytoplasmic as well as membrane antigen could be detected in these assays.

M195 was found to bind specifically only to myeloid cell lines and monocytic cells. Lymphoid cells, including peripheral blood T and B cells, lymph node, spleen, and bone marrow cells, T and B cell lines representing pre-B, early B, B, and late B cell stages and T cell leukemias, and activated fresh B and T cells, did not express the M195 antigen. Red cells and platelets were also negative. Among 95 nonhematopoietic cell lines and nonhematopoietic tissues, only trophoblasts were reactive with M195. This activity appeared to be cytoplasmic. The presence of myeloid antigens in the cytoplasm of choriocarcinoma cells but not normal trophoblast has been reported (37 Ref.b), but its significance is unknown.

With the myelomonocytic lineage, the distribution of M195 antigen was even further restricted. Polymorphonuclear leukocytes were not reactive nor could significant binding be demonstrated in normal bone marrow mononuclear cells. A small percentage of cells from the peripheral blood mononuclear cells from patients with chronic myelogenous leukemia were positive. These samples contain largely granulocytic percursors up to the band stage. The lack of reactivity with polymorphonuclear leukocytes and

this slight reactivity with CML suggests that the vast majority of mature and precursor myeloid cells do not express M195 antigen. In contrast, myeloid leukemia lines and fresh myeloid leukemias were strongly positive. Cell lines representing the earliest myeloid cells or erythroid cells were either negative or less positive than the myeloid cell lines representing later leukemias. These data place the M195 antigen expression to cells in the early to middle part of myeloid differentiation: the antigen is not present at first and is lost as the cells mature toward granulocytes.

Among monocytic cells, M195 reacted with both monocytic leukemia lines and a fraction of mature peripheral blood adherent cells. It was present on the HL60 variant, IF10, and in reduced amounts after monocytic differentiation of IF10 with vitamin D3 and phorbal esters. Likewise, AMOL blasts contained about 10,000 sites (30) compared to macrophages with 5000 sites. Therefore, like its expression on granulocytic precursors, the expression of the M195 antigen or monocytic cells appears to be maturation dependent.

Analysis of quantitative binding to HL60 cells gave an avidity of binding of the M195 IgG of 3×10^9 liters/mol. Binding was saturable and cell number dependent. These data showed that positive cell lines expressed about 10,000 antigen sites per cell. Therefore, M195 was rather weakly expressed compared to many other cell surface antigens. Although we have been unable to identify the target antigen of mAb M195, several of its features suggest it is a polypeptide. The antigen is heat labile; there are small numbers expressed on the surface. The antigen is rapidly modulated after antibody binding, and the antigen was detected by an IgG2a (which is this laboratory rarely identify carbohydrate).

The extremely restricted expression of this antigen among the cell types tested, the biochemical features noted above, including rapid modulation and internalization, and the small number of sites per cell all suggested that the M195 target may be a receptor important in growth and differentiation of myeloid progenitors. However, studies of the effects of M195 alone on the growth of myeloid cell lines, peripheral blood mononuclear cells (data not shown), and colony forming units (30 Ref.b) have not so far shown any stimulating or inhibiting effects of the mAb.

mAbs reactive with restricted myeloid antigens may be useful in a least four areas.

A) Study of the Growth and Differentiation of Myeloid and Monocytic Cells. Of the many antigen and antibody systems that have been described in myelomonocytic differentiation, three systems which have defined different states of myeloid maturation have been most widely studied: the CD34 system (mAbs MY10, 12.8, 3C5) (19–21 Ref.b) which identifies a gp115 found on the earliest hematopoietic progenitors, both lymphoid and myeloid, and which rapidly disappears upon differentiation is also found on some non-hematopoietic tissues including endothelium. mAbs to this antigen have been used to purify progenitors for reconstitution of bone marrow (18 Ref.b). The CD33 antigen system (mAbs MY9, L4F3, L1B2) (24–27 Ref.b) identifies a gp67 (17 Ref.b) restricted to early myeloid and monocytic cells. It is absent from the earliest hematopoietic progenitors and other normal tissues and has been used to eliminate leukemia cells, while sparing the ultimate progenitors, from bone marrow. The CD15 antigen system (multiple mAbs) identifies the Lewis X antigen found on granulocyte colonies from the day 7 stage on and increases expression as cells mature to the polymorphonuclear cell. The antigen is also widely distributed throughout normal tissues (38 Ref.b).

The distribution of the M195 antigen detailed in this paper shows it to fall into the myelomonocytic-restricted second category. Competition binding studies and binding to CD33 transfectants (discussed in Experiment 2 and 30 Ref.b) demonstrated that M195 was carried on the CD33 protein. However, cotyping on fresh leukemias showed that the antigen detected by mAb M195 was not identical to the other CD33 antigens (30 Ref.b).

B) Diagnosis of Hematopoietic Neoplasms. mAbs useful in diagnostic applications must be lineage specific, but not necessarily stage specific. For this reason, the CD34 antigen which is also present on lymphoid cells is less useful than the myelomonocytic antigen systems CD13 and CD15 or the monocytic specific antigens CD14 (27, 39, 40 Ref.b). M195 was restricted to myelomonocytic cells and is useful in the diagnosis of ANLL.

C) Purging of ANLL from Bone Marrow. In order to be useful in bone marrow purging, in addition to being myelomonocytic specific, the mAb must spare the ultimate progenitor cell. Reactivity with other tissues outside of the bone marrow is not important. The ability to fix complement is important but new methods to kill cells with toxins (41 Ref.b) or remove them with magnetic beads (42 Ref.b) may reduce this requirement. CD15 antibodies have proven most useful in this application and are in clinical trials currently (12 Ref.b). CD33 antibodies may be even more useful, if adequate recovery of the bone marrow progenitors can be assured. M195, which rapidly and efficiently kills leukemic cells with rabbit complement, can be successfully applied to this problem. (Lemoli R. M., Gulati S. C., Scheinberg D. A., Gasparetto C., Moore M. A. S., Clarkson B. D., Gee T., Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia (AML): In Vitro Treatment with Myeloid-Specific Monoclonal Antibodies (MoAbs) and Drugs in Combination. Blood 74(7):suppl,p280a abstract) (1989) see Experiment 5). Because the antigen can also be found on clonogenic neoplastic cells, the mAb can also be used to treat lymphoid neoplasms (Hudson, Anne-Marie, Makrynikola, V., Kabral A., Bradstock K. F., Immunophenotypic . . . Blood, 74(6):2112–2120 (Nov. 1, 1989).

D) Therapy with mAb in Vivo. This application is most difficult as it optimally requires limited reactivity with normal tissues, in addition to the criteria described above. Of the many antigen systems described for myelomonocytic cells, CD33 appears most suited for this application in vivo. M195 may be used in application, but its demonstrated lack of cytotoxicity in the presence of human complement or PBMC in vitro might require that the mAb carry a cytotoxic isotope or toxin to be effective. Since the antigen and antibody are rapidly internalized, this therapeutic modality is feasible and investigations of this application have now demonstrated delivery of antibody and conjugated isotope to leukemia cells in the blood and bone marrow (Scheinberg D. A., Lovett D., Divgi C. R., Berman E., Finn R., Graham M. C., Pentlow K., Clarkson B. D., Gee T. S., Larson S. M., Oettgen H. F., Old L. J., A Phase I Trial of Monoclonal Antibody M195 (Anti-CD33) In AML: Pharmacology, Toxicity, Radiolocalization, Dosimetry, ASCO Abstract, to be published in May, 1990) (Protocols). (Hudson, Anne-Marie, Makrynikola, V., Kabral A., Bradstock K. F., Immunophenotypic . . . Blood, 74(6):2112–2120 (Nov. 1, 1989).

EXPERIMENT 2

(Ref.a)

In experiment 1 we described a mouse monoclonal antibody, M195, which detects an antigen found on early

myeloid cells, monocytes, and ANLL cells but not on cells of other hematopoietic or nonhematopoietic lineages (16 Ref.a). The antigen described has several features in common with the myelomonocytic antigen CD33 (4,5 Ref.a) which is found on early myeloid cells and ANLL cells but not on the ultimate progenitor cells (17 Ref.a), a characteristic which may allow selective killing of ANLL cells (18 Ref.a). In this study, we describe the specific reactivity of M195 with ANLL among 227 different fresh hematopoietic neoplasms. The reactivity was similar but not identical to that of MY9 (CD33). Cross-blocking of the binding of these two antibodies to target cells was found. In combination with MY9, M195 showed specificity in diagnosing ANLL by flow cytometry of clinical specimen. M195 bound to most CFU-GM, as measured by colony forming assays. This pattern of reactivity of M195, together with its lack of reactivity with adult tissues (16 Ref.a) make mAb M195 useful in therapeutic trials in humans.

MATERIALS AND METHODS

Monoclonal Antibodies. M195, a mouse IgG2a, was prepared in this laboratory as described in the Experiment 1 (above). The following mAbs were purchased from Coulter Immunology (Hialeah, Fla.): MY9, an IgG2b, (CD33); B4, and IgG1 (CD19); B1, an IgG2A (CD20); I_2 or I_3, IgG2As (anti-HLA-DR); MY4, an IgG2b (CD14); and MY7, an IgG2b (CD13). These were either obtained as fluorescein isothiocyanate conjugates or pure immunoglobulins. The following mAbs were purchased from Becton-Dickinson (Mountain View, Calif.): MY10, an IgG1 (CD34) and goat anti-mouse Ig fluorescein isothiocyanate conjugate of F(ab) '2. L4F3, IgM (CD33) ascites was the gift of Dr. Irwin Berstein. M31, IgM, (CD15) and OKB7, IgG2B (CD21) from a hybridoma provided by Ortho Biotech (Raritan, N.J.) were prepared in this laboratory.

Flow Cytometry. Five million fresh live mononuclear cells from bone marrow or blood from patients on the Hematology-Lymphoma Service at Memorial Hospital were incubated in 0.1 ml final volume with the fluorescein conjugated monoclonal antibodies for 30 min at 4° C. and then washed twice and fixed with 0.1% paraformaldehyde before analysis. For indirect immunofluorescence, after the primary antibody incubation for 30 min at 4° C., 50 μl of goat anti-mouse fluorescein conjugate were added for 30 min, followed by washing and fixing. In some samples, whole blood was analyzed by direct immunofluorescence using the Q prep method (Coulter). Ten thousand cells were analyzed on either an EPICS C or an EPICS profile (Coulter) flow cytometer. Blasts were gated for analysis. Samples containing greater than 25% positive cells (using an isotype matched control Ig to designate the upper limit of negative fluorescence intensity) were scored as positive.

Radioimmunoassays. M195 IgG2a was purified by protein A affinity chromatography, radiolabeled with iodine-125, and used in direct radioimmunoassays on live leukemia and bone marrow cells as described before (16 Ref.a). M195 was labeled to 2–10 μCi/μg protein. Specific binding was determined by subtracting the amount of M195 IgG2a bound in the presence of an excess of unlabeled M195 IgG2a. Non-specific binding was about 400 pg per million cells (1600 sites per cell). Binding at this level or below was therefore considered insignificant.

Morphological Designation of Leukemias. Acute leukemias in patients on the Leukemia Service at Memorial Hospital were classified according to the French-American-British (FAB) criteria (19 Ref.a) and were reviewed by at least one of the authors. Undifferentiated cells with negative histochemical stains which did not appear to be lymphoid and which did not meet FAB criteria for other diagnoses were classified as M0 (two cases only). Bone marrow aspirates and peripheral blood smears were stained with McNeil's tetrachrome (Polyscience, Warrington, Pa.) for morphology. Histochemical analysis included staining with Sudan black B and/or peroxidase and periodic acid Schiff, alphanaphthylbutyrate and ASD chloroacetate esterase, acid phosphatase, terminal deoxynucleotidyl transferase (Tdt). Potential B cell neoplasms were analyzed by mouse red cell rosetting and by indirect immunoperoxidase for immunoglobulin products. The presence of the sheep red blood cell receptor on T cells was determined by rosetting at 37° C. and 4° C. (and by monoclonal antibody by flow cytometry).

Determination of Bone Marrow Colony-forming Units. Bone marrow mononuclear cells were assayed for colonies derived from CFU-GM, CFU-GEM, and BFU-E as described (13 Ref.a). Cultures consisted of Iscove's modified Dulbecco's medium (Gibco, Grand Island, N.Y.) with 24% fetal calf serum, 0.8% deionized bovine serum albumin (Sigma Chemical, St. Louis, Mo.), 10^{-4}M 2-mercaptoethanol (Sigma Chemical), 1 U partially purified human urinary erythropoietin (49 U/mg) (Toyobo, New York, N.Y.), 10% MO T cell line conditioned media, and 1.3% methylcellulose. Cultures were prepared in quadruplicate and scored on days 7 or 14. In some assays, adherent cells were depleted first by plastic adherence at 37° C. for 90 min.

Antibody mediated complement cytotoxicity of colony forming units was determined by incubating the marrow mononuclear cells first in excess monoclonal antibody (10–100 μg/ml) and low toxicity baby rabbit complement (Pel freeze), at a final dilution of 1:8, for 30 min at 37° C. followed by two washes with media. Alternatively, human serum was used as a complement source.

Preparation of Purified Normal Progenitor Blasts. Normal bone marrow cells were depleted of accessory and maturing cells to obtain enriched populations of progenitors by negative selection using density separations and a panel of monoclonal antibodies followed by immune rosetting or panning as described (13 Ref.a). The 12 antibodies reacted with cell surface antigens present on mature T, B, myeloid, and monocytic cells. Cells were then frozen in liquid N_2, and thawed once, then reseparated on Ficoll-Paque (Pharmacia, Piscataway, N.J.) before use.

RESULTS

Distribution of M195 on Hemstopoietic Neoplasm. The binding of mAb M195 to mononuclear cells from 227 patients as measured by flow cytometry is shown in Table 1. M195 was found on the majority of myeloblastic leukemias; 80% of the positive ANLL cases had greater than 50% of cells positive for M195. Forty percent of positive cases had greater than 75% of cells positive for M195. Lymphoid leukemias, lymphoproliferative disorders, and other nonmyeloid samples were virtually always negative (4% of cases positive).

A quantitative analysis of the total number of binding sites on several of the positive hematopoietic neoplasms was conducted by radioimmunoassay. Our accompanying study (16 Ref.a) showed that myelomonocytic leukemia cell lines expressed approximately 10,000 antigen sites per cell. The same quantity was seen on fresh ANLL cells from several patients (FIG. 6). Lymphoid leukemias and lymphomas, and chronic myclogenous leukemia (CML) cells in chronic phase did not express antigen on their surfaces.

TABLE 1

Distribution of M195 on Hematopoietic Neoplasms By Cytometry*

	No. Tested	No. Positive	(%)
Acute nonlymphoblastic leukemia	54	34	(63)
Tdt-positive cases only	10	3	(30)
Chronic myelogenous leukemia-			
Accelerated and myeloblastic phase	7	7	(100)
Total myeloid, blastic cases	61	41	(67)
Chronic myelomonocytic leukemia	3	3	(100)
Myelodysplastic syndromes	25	12	(40)
Chronic myelogenous leukemia (chronic)	17	7	(41)
Acute lymphoblastic leukemia			
Calla+	33	4	(12)
Calla–	8	0	
T-ALL	5	0	
Chronic myelogenous leukemia-			
Lymphoblastic phase	5	0	
Total lymphoid, blastic cases	51	4	(8)
Lymphoproliferative disorders (T + B)	19	1	(5)
Normal, nondiagnostic, and other	51	0	
Total cases	227		

*Conducted as described in the Materials and Methods

The expression of M195 was compared to the FAB classification of ANLL (Table 2). M195 was expressed in all subclasses of ANLL except M0 and M6. Since there were very few leukemias of these two classes, the significance of this is not clear. However, when both the M0 and M1 classes were pooled, only 3 of 14 (23%) were positive compared to 30 of 44 M2, M3, or M4 leukemias (68%).

TABLE 2

Distribution of M195 Antigen among FAB Morphological Subgroups of AML

FAB Group	No. Tested	No. M195-Positive	(%)
M0	2	0	(0)
M1	12	3	(25)
M2	24	15	(63)
M3	5	5	(100)
M4	15	10	(67)
M5a	5	3	(60)
M5b	18	8	(44)
M6	2	0	(0)
M7	1	1	(100)

Tdt-positive ANLL (>320 ng/10^8 cells) also tended to be M195-negative (30%) compared to the Tdt-negative myeloid leukemias (74%). These data supported the suggestion from our earlier paper on cell lines that the M195 antigen was more highly expressed on early committed granulocytic precursor cells than on more undifferentiated earlier myeloid cells (16 Ref.a).

M195 was found on about a third of CML samples in chronic phase, all CML samples in myeloblastic or accelerated phase but not on lymphoblastic CML cells (Table 3A). Four of 46 acute lymphocytic (ALL) leukemias were M195-positive (Table 3B). These ALL samples were CALLA-positive pre-B leukemias. The total number of cells positive for M195 in these samples was rather low: 26%, 32%, 39%, and 42%. Other markers are shown for comparison. MY9 was present on five pre-B leukemias, with 27%, 28%, 35%, 35%, and 62% MY9-positive cells; four of these were different from those that were M195-positive. One case was 28% MY9-positive and 42% M195-positive. MY9 was also found on one of five cases of lymphoblastic CML (Table 3A).

TABLE 3A

Immunophenotype of Chronic Myelogenous Leukemia at Memorial Hospital

	No. tested	M195	My9	My10	My7	My4	Calla/B4	Ia
CML chronic phase	24	17	41	41	19	18	6	6
CML accelerated and myeloblastic 7	7	100	70	100	86	28	ND	86
CML lymphoblastic	80	5	0	20	100	50	0	80

TABLE 3B

Immunophenotype of Acute Lymphoblastic Leukemias at Memorial Hospital*

ALL type	No. tested	Ia	My10	B4	B1	M195	My9
Ia							
CALLA+	33	82	58	91	52	12	15
CALLA–	8	88	88	100	0	0	0
T-ALL	5	0	0	0	0	0	0
Total non-T ALL		41	83	64	95	41	1012
Total ALL	46	74	57	85	37	9	11

*Conducted as described in Materials and Methods

A Comparison of M195 with CD33 Antigens. Our previous paper suggested that the distribution of M195 appeared similar to that described for CD33-reactive antibodies MY9 and L4F3. The protein target of M195 has thus far eluded detection (16 Ref.a). A comparison of M195 reactivity to other well characterized myeloid markers on the same leukemias is shown in Table 4 (this table does not include 30 leukemias that were not characterized by other markers that were included in Table 2). MY10, MY7, and MY4 were distributed among all subtypes in patterns dissimilar to M195. MY9 was strikingly similar to M195 in its pattern of distribution. An analysis of the concordance of M195 and MY9 in the flow cytometric studies on fresh, acute blastic leukemias (lymphoid and nonlymphoid) is shown in Table 5. In 93 cases of ANLL or acute lymphoid leukemias, either both markers were positive or both were negative. In 19 cases the binding differed, resulting in a concordance rate of 83% overall. This high, but not complete, concordance suggested that the M195 antigen might be related to or coexpressed with the CD33 antigen.

TABLE 4

Immunophenotype of FAB Subgroups of AML at Memorial Hospital*

Group	No. tested	% Testing Positive for: My10	My7	My9	M195	My4
M0	2	100	100	0	0	0
M1	5	80	50	60	40	0
M2	15	67	33	73	73	7
M3	4	100	50	100	100	75

TABLE 4-continued

Immunophenotype of FAB Subgroups of AML at Memorial Hospital[a]

Group	No. tested	% Testing Positive for: My10	My7	My9	M195	My4
M4	10	70	40	80	70	44
M5a	3	100	33	100	67	0
M5b	12	58	45	75	58	17
M6	2	50	100	50	0	0
M7	1	100	0	100	100	0

[a]Conducted as described in Materials and Methods

TABLE 5

Concordance Data for M195 and My9 among 112 Blastic Leukemias

Reactivity Pattern	No. of Cases
My9 + and M195+	38
My9 − and M195−	55
My9 + and M195 − or My9 − and M195+	19
Over all concordance	83%

Cross-blocking experiments using iodine-125-labeled M195 IgG or F(Ab')2 binding to HL60 leukemia cells in the presence of excess concentrations of various immunoglobulins are shown in FIG. 2. Both MY9 and L4F3 (CD33-reactive), as well as the original M195 IgG, blocked binding of the ^{125}I-M195. MY7 (CD13), M31 (CD15), and OKB7 (CD21) did not inhibit binding. These data further confirmed the association between the M195 antigen and CD33 antigens. In other experiments (not shown) excess unlabeled M195 was able to block binding of about 50% of FITC-labeled MY9 to HL60 cells as measured by flow cytometry.

Mab M195 was also tested by Dr. T. Look (St. Jude, Memphis, Tenn.) for reactivity with NIH-3T3 cells transfected with the DNA from myeloid cells and expressing the CD33 antigen (20 Ref.a). Both L4F3 (CD33) and MY9 (CD33) are reactive with these cells; M195 was reactive as well. This result, when taken in context with the nonidentical concordance data shown above, suggested that the M195 antigen was carried on the p67 (CD33) but was not the same as the previously described CD33 antigen epitopes recognized by L4F3 and MY9.

Diagnostic Utility of M195. MY9 is widely regarded as the standard marker for ANLL (21,22 Ref.a). We compared the diagnostic utility of MY9 with M195 either alone or together on 81 blastic leukemias of either myeloid or lymphoid origin (Table 6). Eighty-four percent of ANLL expressed either M195 or MY9, but each antibody alone failed to identify more than a quarter of cases. Among lymphoid cases either MY9 or M195 was occasionally expressed, but both antibodies were expressed together only once. In this case the reactivity was weak: 28% MY9+ and 42% M195+. Thus, the presence of both M195 and MY9 positivity on a leukemia sample had 98% specificity in defining that leukemia as ANLL.

TABLE 6

Diagnostic Utility of M195 and My9 among Blastic Leukemias

Positive Cases with the Indicated Marker(s)

My9	M195 Alone	Both M195 and My9 Alone	Either M195 and My9
Sensitivity[a] in 61 Myeloblastic cases	67%	74%	67% 8%
Specificity[a] in 51 lymphoblastic cases	8%	12%	2% 20%

[a]Antibody(s) should be positive in all cases
[b]Percent of cases positive by flow cytometry
[c]Antibody(s) should be negative in all cases

Expression of M195 on Hematopoietic Colony-forming Cells. The expression of M195 on leukemia cells, but not on mature nonadherent peripheral blood cells nor on any detectable nonadherent bone marrow cells (16 Ref.a), suggested that M195 might be expressed on a small group of hematopoietic progenitors. The expression of M195 antigen on hematopoietic progenitors was studied by analyzing the recovery of bone marrow colonies after treatment of bone marrow mononuclear cells with M195 and rabbit complement (Table 7). Complement alone, antibody alone, and no antibody or complement treatments were used as negative controls. Antibody to human IA antigen (gift of Dr. J. D. Griffin) was used as a positive control. The number of CFU-GEMM recovered was not sufficient to obtain statistically significant data. In three of four experiments, M195 and complement eliminated almost all of the 14-day CFU-GM; burst forming unit erythrocytes were also killed, although the average recovery was somewhat higher.

TABLE 7

Recovery of Colonies after Treatment with Antibody and Complement

Treatment	Day 7	% Recovery of the Following Colonies: Day 14 CFU-GM	CFU-GM/BFU-E
Nil	124[a] (111,136)	126(139,104,111,110)	103(143,83115;71),
M195 alone	124(126,181,93,98)	108(97,120)	107(141,114,98,74)
Complement alone		100[c]	100100
M195 plus complement	33(8,77,6,40)	6(10,1)	17(0,60,3,6)
Anti-IA plus complement	6(5,11,1,ND	0(0,ND[d])	1(0,2,0,ND)

[a]Mean of all experiments shown
[b]Percent recovery of an individual experiment
[c]The "complement alone" treatment was considered to be 100% recovery, and other data on this chart were normalized to that value. Plating efficiency was between 0.10 and 0.15 percent
[d]ND = Not determined.

In order to determine the extent of expression of M195 on early hematopoietic progenitors, radioimmunoassays were conducted on highly purified blasts. The cells used were isolated by negative selection with a panel of 12 monoclonal antibodies and immunorosetting or panning followed by freezing and thawing once (13 Ref.a). These cells are morphologically blasts and represent a progenitor cell population 50–100-fold purer than bone marrow mononuclear

cells. Five to fifteen percent of these cells typically form myeloid and erythroid colonies.

No binding of ^{125}I-M195 above background was found in testing three different samples of these normal, early blast cells. A small percent of positive cells could escape detection using this assay.

Because these data suggested that M195 antigen was expressed on a minor population of bone marrow cells responsible for CFU-GM colonies, we attempted to identify these cells by positive selection with panning, immunomagnetic bead separation, affinity sepharose bead separation, and fluorescence-activated cell sorting. None of these methods selected out a M195-positive subpopulation. This may be due to weak antigen expression, antibody affinity, or other unknown problems.

Effects of M195 on Bone Marrow progenitors in the Presence of Human Complement. Because we anticipated possible use of M195 in vivo for therapy of ANLL, we studied the effects of M195 on CFU-GM and 5FU-E from normal bone marrow in the presence of human serum as a complement source (Table 8A). No killing of CFU-GM or BFU-E was seen at 14 days. The effect of the continued presence of M195 in bone marrow culture was also studied by adding the antibody to the methyl cellulose at days 1 and 5 after plating, with no added complement (Table 8B). These experiments were done to determine if the antibody had a growth stimulatory or inhibitory effect on progenitor cells in the marrow. No effects were seen. Similar growth studies of peripheral blood mononuclear cells and HL60 leukemia cells were also negative.

TABLE 8A

Effects of M195 on Hematopoietic Stem Cells in the Presence of Human Complement

	No. of Colonies, Day 14	
Treatment	CFU-GM	BFU-E
None	116(100%)[b]	154(100%)
Complement (C) alone		102(100%)121(100%)
M195	132(113%)	158(102%)
M195 + C[c]	141(138%)	127(105%)

TABLE 8B

Effect of the Continued Presence of M195 on Colony Forming Cells[a]

Nil	125(100%)	188(100%)
M195	124(99%)	168(89%)

[a]Human serum was added at a final dilution of ⅙ as described for rabbit complement in Materials and Methods
[b]Quadriplicate control plate results are normalized to 100%
[c]M195 IgG was added directly to growing cultures as described in Materials and Methods

DISCUSSION

This paper describes the distribution of mAb M195's binding on fresh leukemia cells and early hematopoietic progenitors. In our accompanying paper (16 Ref.a) we showed that the M195 antigen was present on myelomonocytic leukemia cells and a fraction of monocytes but was not detectable on more mature myeloid cells present in the bone marrow or peripheral blood nor on nonhematopoietic cells and tissues. In this paper we extend the description of the M195 antigen and directly compare it to other well-characterized myeloid and monocytic antigens. Among 227 fresh hematopoietic samples studied, M195 antigen expression was largely restricted to differentiated ANLL. Undifferentiated and Tdt-positive ANLL were less likely to display antigen. However, FAB classification did not correlate specifically with M195 expression.

Quantitative analysis by radioimmunoassay showed that about 10,000 sites were expressed on the cell surface of ANLL cells. Our studies (16 Ref.a) have demonstrated rapid modulation of these sites after antibody binding.

Several antigens are currently used to diagnose ANLL by flow cytometry. Among these, CD33 antibodies, MY9 (5 Ref.a), and L4F3 or LIB2 (4 Ref.a), appear to be most widely and most specifically distributed on ANLL. M195 antigen was concordantly expressed with MY9 on 83% of cases. Moreover, although neither antigen was expressed on 100% of ANLL, the combination of both M195 and MY9 could be used to diagnose ANLL with 98% specificity if both were expressed on a leukemia sample. We are currently using this combination to aid in the diagnosis of acute leukemias at Memorial Hospital.

The close coexpression of M195 and MY9 suggested that M195 might bind to the CD33 protein target [p67] (20 Ref.a). Efforts to identify the M195 target have been unsuccessful (16 Ref.a). Blocking experiments shown here demonstrated probable identity of the M195 target with the CD33 protein. Moreover, binding of M195 and CD33 DNA transfectants was shown. Despite these data, since flow cytometry data showed nonidentical concordance with MY9, it is likely that M195 does not bind to the same CD33 epitope as MY9 or L4F3.

Although M195 antigen was found on a greater percentage of ANLL samples of the FAB classifications M2, M3, and M4 than on other types, the presence of M195 binding could not be used to predict morphology or vice versa. Other studies comparing immunophenotype with morphologic phenotype have come to similar conclusions since there was considerable overlap of markers into each type of ANLL (23–25 Ref.a). Some discrimination of monocytic from myeloid ANLL has been shown (22,26 Ref.a), however.

The CD33 antigen is expressed on early myelomonocytic progenitor cells (4,5 Ref.a), but not on the ultimate progenitors (17 Ref.a). This restriction has allowed selective purging of ANLL cells from bone marrow while still permitting regrowth of normal cells in selective cases (18 Ref.a)(Lemoli R. M., Gulati S. C., Scheinberg D. A., Gasparetto C., Moore M. A. S., Clarkson B. D., Gee T., Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia (AML): In Vitro Treatment with Myeloid-Specific Monoclonal Antibodies (MoAbs) and Drugs in Combination. Blood 74(7):supp1,p280a abstract) (1989) see Experiment 5). M195, as expected, was expressed on CFU-GM and to a lesser extent on BFU-E. Since, like MY9 and L4F3, M195 readily kills cells with rabbit complement, it is useful as a purging agent in ANLL. Radioimmunoassays with M195 on highly purified early blasts did not detect significant antigen expression. Because the radioimmunoassay could miss M195 expression on small subpopulations within this group of cells, long-term marrow cultures were done (Lemoli R. M., Gulati S. C., Scheinberg D. A., Gasparetto C., Moore M. A. S., Clarkson B. D., Gee T., Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia (AML): In Vitro Treatment with Myeloid-Specific Monoclonal Antibodies (MoAbs) and Drugs in Combination. Blood 74(7):supp1,p280a abstract) (1989) see Experiment 5) to help further define and confirm

this finding. Based on the data here and in Experiment 1 (16 Ref.a) the distribution of the M195 antigen among hematopoietic differentiation appears similar to that described for other CD33 antigens (4.5 Ref.a). This includes early committed myeloid progenitors, but not the earliest colony forming cells (Lemoli R. M., Gulati S. C., Scheinberg D. A., Gasparetto C., Moore M. A. S., Clarkson B. D., Gee T., Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia (AML): In Vitro Treatment with Myeloid-Specific Monoclonal Antibodies (MoAbs) and Drugs in Combination. Blood 74(7):supp1,p280a abstract) (1989) see Experiment 5) (17,18 Ref.a).

The M195 antigen is not expressed on adult human tissues. Therefore, in addition to its use as a diagnostic marker of ANLL and as a purging agent, M195 can potentially be used as a therapeutic agent in vivo. Since the antibody does not have in vitro cytotoxic effects alone or in the presence of human serum as a complement source, it is not likely to cause lysis of ANLL cells. However, upon binding of mAb M195, the antibody is rapidly internalized (Digi, Internalization of Radionuclides (Experiment 3)), and thus the application of mAb M195 as a carrier of toxins or isotopes to ANLL cells in vivo is feasible.

EXPERIMENT 3

In this experiment, we focus on the impact of modulation and mAb internalization and release on the delivery of radionuclide into tumor cells. We investigated the behavior of two prototype radionuclides, radioiodine and radioindium, when attached to mAbs currently under evaluation at Memorial Sloan-Kettering Cancer Center in Phase I clinical trials.

Iodine-125 serves as a prototype for all halides such as ^{77}Br, ^{123}I, ^{131}I, ^{211}A, and ^{124}I. ^{111}In serves as a prototype for all radiometals such as ^{211}Bi, ^{25}Tb, ^{90}Y, ^{186}Rh, and ^{188}Rh. The mAbs modulate subsequent to interaction with antigen. Mab M195 is reactive with a 67 KD cell-surface glycoprotein found on most myeloid leukemia cells (24,25 Ref.c), and is reactive with the Epstein Barr Virus (EBV) receptor, a 140 KD glycoprotein surface receptor expressed on most B-cell lymphoma cells and chronic lymphocytic leukemia (26,27 Ref.c). In addition, we explored the behavior of a proteolytic digestion fragment of the antibody.

MATERIALS AND METHODS

Cells:

HL60, a myeloid leukemia cell line, were maintained in log phase growth in RPMI 1640 supplemented with 10% FCS and 10% l-glutamine at 37° C. in 5% carbon dioxide. Cells were utilized only if the viability (as estimated by trypan blue exclusion) was 95% or greater.

Antibodies:

M195 is an IgG2a monoclonal antibody (mAb) reactive with a 67 KD cell-surface glycoprotein found on most myeloid leukemia cells (24,25 Ref.c). The antibody was purified from mouse ascites fluid using Protein A affinity chromatography. M195 F(ab')$_2$ was prepared by pepsin digestion of the intact immunoglobulin; OKB7 Fab was prepared by papain digestion.

Radioiodination:

Intact antibodies and their fragments were labeled with iodine-125 by the chloramine-T method (28 Ref.c). 100 ug of antibody was incubated with 2 mCi of ^{125}I and 20 uL of a freshly prepared solution of chloramine-T at a concentration of 2 mg/mL in 0.2M phosphate at pH 7.4 for 1 minute at room temperature. The reaction was quenched with 20 uL of a freshly prepared solution of sodium metabisulfite at 20 mg/mL and incubated for 5 minutes. The radiolabeled antibody was purified by exclusion chromatography (Sephadex G25, Pharmacia Inc., Piscataway, N.J.). Further purification of the mAb from free radionuclide was effected by dialyzing the radiolabeled mAb at 4° in phosphate buffered saline (PBS), pH 7.4.

Immunoreactivity of the labeled antibody (that proportion of antibody molecules in a preparation which were capable of binding to antigen) was determined by modifications of previously described methods (28,29 Ref.c) as follows: 10^7 cells of at least 95% viability at 4° C. were incubated with 5 ng of the radiolabeled antibody for 60 minutes. The percent bound was estimated; the supernatant obtained after centrifugation was transferred to a similar set of cells and the process repeated until binding was no greater than with a control cell line. Immunoreactivity was not less than 65% for M195.

^{111}Indium Labeling:

The intact immunoglobulins were conjugated with diethylene triamine pentaacetic acid (DTPA) as described (31 Ref.c). After conjugation, each antibody was labeled with ^{111}In by incubation with ^{111}In at a pH of 3.0 for 60 minutes at room temperature. The reaction was quenched by increasing the pH to greater than 6.5 using 0.2M metal-free phosphate buffer (pH 7.4). The radiolabeled mAb was purified using a Chelex metal-binding column followed by size exclusion chromatography. Further removal of free ^{111}In from mAb was accomplished by dialyzing the radiolabeled mAb at 4° in metal-free PBS, pH 7.4. Immunoreactivity of the labeled antibody was comparable to that of the respective radioiodinated antibody.

Radionuclide Internalization

5 million viable cells in 5 mL media were incubated with 5 ug radiolabled antibody, at either 4° or 37°. Immediately after addition of the radiolabeled mAb (or fragment) and at several times afterward, 200 uL aliquots from each batch of cells were taken and washed three times. 1.5 mL glycine/sodium chloride (50 mM glycine/HCl, 150 mM sodium chloride, pH 2.8) was added to each pellet. "Total" cell-associated radioactivity was determined in a gamma counter after mixing. The cells were then centrifuged, the supernatant aspirated, and the cell pellet re-counted to determine "internalized" radioactivity. "Cell surface" radioactivity was calculated as the difference between total and internalized radio-activity. This general method has been used to study internalization of other cell surface receptors(20-22 Ref.c). We have confirmed that this direct method measures true losses of antibody from the surface by indirect methods (indirect radioimmunoassay and complement fixation) as well. The percent radioiodine bound to protein in the supernatant was estimated by TCA precipitation of the supernatant; it was never less than 95%. Similarly, protein-bound radioindium was estimated in aliquots of selected supernatants by thin layer chromatography and was always greater than 90%.

Radionuclide Release:

5 million viable cells in 5 mL media were incubated with 5 ug radiolabeled mAb or fragment at 4° for 60 minutes. The incubated cells were then washed two times in media and resuspended to the same volume. Baseline total and internalized radioactivity was determined as described above. Immediately after washing, the washed cells were separated into two parts and kept at either 4° or 37°. Total cell-associated radioactivity and internalized radioactivity were then determined over time as described above.

RESULTS

All experiments were done 2–6 times and all time points were done in duplicate. The mean of two determinations was

recorded. Maximal binding of M195 was about 10,000 sites per cell; for OKB7, about 100,000 sites per cell.

Internalization experiments were designed to study changes in the kinetics of binding and cell-associated radioactivity in the presence of excess ambient antibody, a condition which might occur during an antibody infusion in patients. The release experiments were designed to study the same phenomena, after a period of binding, in the absence of ambient antibody which might mimic conditions in vivo following termination of mAb infusion.

125I-M195 Internalization and Release: (FIGS. 8A–D)

At 4° , there were initial increases in the total amount of cell-associated radioactivity, with minimal internalization of ^{125}I (FIG. 1A). Bound ^{125}I increased about 4-fold and plateaued by 2 hours, suggesting completion of binding and saturation of sites within this time period. At 37°, there was a similar increase in total cell-associated radioactivity over time as at 4°. In contrast, there was a significant greater increase in the amount of internalized radioactivity over time, with most of the increase being noted in the first two hours. After two hours, the internalized radioactivity stabilized at about a 12-fold higher level than at time 0.

The release experiments for M195 IgG (FIGS. 9C–D) showed no significant change in cell-associated radioactivity over time at 4°; the amount of radioactivity in the surface and within the cell did not change over time. At 37°, there was an initial rapid clearance of about 40% of the cell-associated radioactivity. The radioactivity that cleared appeared to be accounted for by net loss from the cell surface. Whether this was direct or via an internalization step cannot be determined from these data. There was an increase in internalized radioactivity with time. Clearance of radioactivity from the cell surface occurred during the first hour, after which the total amount of cell-associated radioactivity stayed constant while the amount of internalized radioactivity increased to plateau toward the end of the experiment.

The present of cell-associated radioactivity that was internalized was constant for cells at 4° (FIGS. 8B, D). At 37° the percent of internalized radioactivity approached 35% of total cell-associated radioactivity in the presence of excess antibody, and up to 60% in the absence of ambient antibody.

In the presence of surrounding antibody there appeared to be no net loss of cell-associated radioactivity, with increases in the amount within the cell contributing to the increase in percent radioactivity internalized. In contrast, in the absence of ambient antibody (FIGS. 8C, D), there was a net loss of total cell-associated radioactivity manifest as a transfer of radioactivity from the cell surface to both the surrounding media and to the cell interior.

This may have occurred either because of internalization followed by release or by an independent direct release of surface-bound radioactivity into the media. This net loss translated to an increase in the percent radioactivity internalized greater than that apparent solely from the increase in internalized radioactive counts. The net result Was that after 2 hours, more than half the radioactivity associated with the HL60 cells was intracellular.

^{111}In-M195 Internalization and Release: (FIGS. 9A–D)

Additional experiments were conducted as above, using ^{111}In-M195 (FIGS. 9A, B). Large increases in internalized radioactivity occurred over time at 37° as compared to 4° (FIG. 9A). Internalization of ^{111}In as a percentage was consistently higher than with ^{125}I, increasing with time, not showing a plateau out to 4 hours, to reach approximately 70% in some experiments. This may be due in part to a greater loss (60%) of total ^{111}In at 37° (FIG. 9C), perhaps by release of chelated ^{111}In from surface-bound IgG. In this as well as the ^{111}In-OKB7 experiments, the background percent internalization was considerably greater than observed with ^{125}I-labeled antibody, perhaps due to transchelation of ^{111}In to cellular protein.

In summary, at 4°, when cells would be expected to be metabolically quiescent, there were no major changes in cell-associated radioactivity over time, whether on or in the cell and whether or not there was surrounding antibody present. In contrast, cell-associated radioactivity changed dramatically over time at 37° when the cells are metabolically active.

^{125}I-M195 $F(ab')_2$ Internalization and Release: (FIGS. 10A-D)

In contrast to the findings observed with the intact Ig, with the $F(ab')_2$ the early increase in total cell-associated radioactivity decreased moderately with time. The internalized radioactivity, however, increased gradually with time at 37° while staying relatively constant at 4°. Background (time 0) radioactivity was consistently higher with the $F(ab')_2$. The increase in internalized radioactivity was not as great over time with the fragment as with the intact Ig (26% to 37% with the fragment compared to 6% to 32% with the Ig).

In the absence of ambient antibody, there was rapid decrease in the total cell-associated radioactivity with time at 37%; this was accompanied by a significant, albeit small, decrease in the total amount of radioactivity within the cell; the percent internalized over time at 37° therefore did not increase as much as with the intact Ig.

Because of differences in specific activity of the labeled Ig and fragments and differences in counting efficiency of the two radionuclides among the different sets of experiments, all changes over time in an individual experiment were related to conditions at time 0 to enable us to compare experiments (Table I). Radioactive counts at time (n) were expressed as a percentage of those at time 0 ($Cpm_{(t=n)}/Cpm_{(t=0)}$33 100). Several conclusions became apparent.

With M195 Ig, an antibody that rapidly internalizes in the presence of ambient excess antibody (the internalization experiments), there was no significant difference between the changes in amounts of radioindium internalized as compared to the changes in amounts of radioiodine internalized (1150% vs. 1300%). In contrast, the percent losses of radioiodine from the cell in the absence of ambient antibody (the release experiments) were considerably less than the losses in amounts of ^{111}In. The relative increase in percent internalized was primarily a result of greater loss of cell-associated radioactivity. Radioiodine attached to the fragment cleared at an even faster rate than that of ^{111}In.

DISCUSSION

As the number of clinical trials employing radioactively tagged monoclonal antibodies for diagnostic imaging or radioimmunotherapy increases, the need for an understanding of the kinetics of antibody binding and internalization of nuclide become increasingly important. Significant differences in binding of ^{111}In labeled antibody compared to radioiodinated antibody have been demonstrated in vitro and in vivo (14–17 Ref.c). The experiments here attempt to describe some of these differences at the cellular level. We show that internalization of radionuclide into target tumor cells is dependent upon the choice of radionuclide, the antigen-antibody system concerned, and the nature of the antibody used, either intact immunoglobulin or fragment.

Recently, Press et al. (20 Ref.c) compared the binding and degradation characteristics of a panel of radioiodinated antibodies reacting against B-cell tumors. As we have also

confirmed here, distinct differences in internalization kinetics were found between mAbs. In addition, we also studied the behavior of intact and fragmented mAbs when labeled with two different prototype radionuclides, ^{125}I and ^{111}In. Significant detachment of radionuclide from antibody was not seen at the early time points we studied. This is similar to results observed by Press and associates. Because steady states were reached within 2–4 hours, we did not examine behavior at late time points.

In the presence of excess surrounding antibody, M195 Ig was rapidly internalized by the cell subsequent to interaction with the target antigen. For both nuclides, there were no differences in binding kinetics (Table I). With the $F(ab')_2$, there were minimal changes in total and internalized radioactivity. After ambient M195 Ig or $F(ab')_2$ had been washed away, there was greater loss of ^{111}n-labeled compared to ^{125}I-labeled Ig, and much greater internalization with the Ig than with the $F(ab')_2$). We do not know if these differences reflect the minor difference in avidity (3×10^9 L/M for the intact Ig compared to 10^9 L/M for the $F(ab')_2$).

Internalization and release kinetics of radionuclide labeled to a mAb that is internalized rapidly were not dependent on the nature of the radionuclide used. Radionuclide attached to the intact M195 Ig showed far greater internalization than when attached to its fragment. Studies with the Fab fragment suggested that lack of modulation and internalization may be more apparent in the absence of excess surrounding antibody.

In a clinical trial using M195 (whether diagnostic or therapeutic), intact M195 Ig might be preferable to the fragment, as a significantly greater amount of radioactivity would then be internalized. The K_D of the $F(ab')_2$ is 10^9 L/M compared to a K_D of 3×10^9 L/M for the intact immunoglobulin; we do not know if the difference in internalization was caused by this small difference in dissociation constants or some other unknown change in antibody binding consequent to creation of the $F(ab')_2$. Although there was greater clearance of ^{111}In from the cell in the absence of ambient mAb, there was also a significantly greater amount internalized. Since in the absence of ambient antibody there is greater loss of cell-associated radioactivity when ^{111}In is the radionuclide used, the ideal radiolabel to be used might either be a suitable isotope of iodine or a radiometal depending upon the physical half-life of the radionuclide and the biological half-life of the antibody in the host.

Although the kinetics of binding, internalization, and release are important, the choice of nuclide for clinical therapy trials would also be affected by the physical half-life, emission characteristics, and cytotoxic potential of the radionuclide under consideration, as well as by labeling characteristics and serum clearance. Dehalogenation of iodine-labeled antibody has been described (17 Ref.c), but current methods of chelation also result in detachment of radiometal from antibody in vivo (17 Ref.c) and human trials have not been uniformly successful to date. Progress in new chelation chemistry may solve these problems (32 Ref.c). Moreover, if radionuclides that decay by electron capture are more cytotoxic when internalized into the cell than those that undergo beta-minus decay, then ^{125}I and ^{123}I would be candidate radionuclides for therapeutic purposes. A recent report by Woo et al. (13 Ref.c) has shown significant cytotoxicity with ^{125}I-labeled antibody compared to unlabeled antibody, postulated to be due to cytotoxicity of intranuclear ^{125}I. We have also seen significantly enhanced growth suppression of ^{125}I-labeled anti-epidermal growth factor receptor antibody compared to ^{131}I-labeled or unlabeled antibody in cells expressing increased quantities of the receptor. We are now studying the cytotoxicity of iodine labeled M195 in vitro.

In summary, the choice of both the radionuclide and the antibody form has great impact on the kinetics of radionuclide internalization and retention in target cells and therefore, may be of crucial importance in the design of clinical trials utilizing radiolabeled antibodies. Preclinical studies of cell binding and internalization such as those described here may help suggest an optimal approach for imaging or therapy.

EXPERIMENT 4

(ASCO, Scheinberg May, 1990, Submitted for Publication)

A Phase I Trial of Monoclonal Antibody M195 (Anti-CD33) in AML: Pharmacology, Toxicity, Radiolocalization

Mouse monoclonal antibody (mAb) M195 reacts with myeloid leukemia cells, early myeloid progenitor cells, some mature monocytes, but with no other adult cells or tissues. A phase I trial using escalating amounts of M195 in 4 daily doses was initiated in patients with relapsed or refractory AML (3 patients per dose level). First doses were trace labeled with iodine-131. After 7 patients (3 at 1 mg/m^2, 4 at 5 mg/m^2), the following has been observed: Rapid, specific targeting to leukemia cells in both blood and bone marrow was observed in all patients within 1 hour as documented by serial blood sampling and bone marrow biopsies and aspirates. Whole body gamma camera images showed uptake in all bone marrow areas and spleen. Efficient targeting occurred in hypoplastic, pancytopenic patients as well as in patients with an estimated leukemic burden in excess of 10^{12} cells, even at the 1 mg dose level. 10^3–10^4 antibodies were delivered per cell within 1 hour with persistence in the bone marrow for several days. The first patient had mild transient generalized pruritis during infusions. One patient developed persistent bone marrow pain after the third dose of 5 mg/m^2. Since M195 is an IgG2A which exhibits no cytotoxicity in vitro using human effector cells or complement, no therapeutic responses were expected or observed yet. Because M195 is rapidly internalized into cells, these data show that rapid, specific delivery of radionuclides, toxins or other immunoconjugates into marrow cells will be feasible with M195.

Monoclonal Antibody Therapy. Several pilot trials have been conducted using unlabeled hematopoietic neoplasms (reviewed in Rosen S T, Zimmer A M, et al. J Clin Oncol 5:562–573, 1987). Toxicity was tolerable and responses were seen, but they were almost always transient. None of the original trials used antibodies with intrinsic cytotoxic capability and responses were probably abrogated by lack of effector functions, antigen modulation and human antimouse Ig responses. More recent trials (Ball E D, Berneri G M, Cornwell G G, et al. Blood 62:1203–12110, 1983; Tanimoto M, Scheinberg D A, Cordon-Cardo C, et al. Leukemia 3:339–348, 1989) using radioisotope-labeled monoclonal antibodies have achieved high major response rates. These trials utilized large doses of Iodine-^{131}-labeled monoclonal antibodies to T-cell lymphomas, chronic lymphocytic leukemias, and nodular lymphomas. The major adverse reactions were hematologic, related to the high doses of radiation. In the trial with T cell lymphomas and CLL, remissions were short lived. It is too soon to know the long term outcome in the nodular lymphoma trials. No study of monoclonal antibodies has been completed with patients with ANLL, but three patients were treated (Scheinberg D A, Tanimoto M, McKenzie S, et al. Leukemia 3:440–445,

1989) with minimal responses and toxicity with a cocktail of antibodies. These antibodies recognized different antigen targets than M195 recognizes. Despite large doses of antibody (up to several hundred mg), blast counts were suppressed only transiently in the three patients. All three patients had fever and one had urticaria due to the infusions, but the antibodies were not highly purified. We have seen no toxicity here in 26 patients with B-NHL, Hodgkins Disease, and T-NHL, treated with monoclonal antibodies OKB7 or R24, at doses ranging from 0.1 to 100 mg.

M195. Mouse monoclonal antibody M195 is an IgG2a developed at Sloan-Kettering Institute (Tanimoto M, Scheinberg D A, Cordon-Cardo C, et al. Leukemia 3:339–348, 1989. Scheinberg D A, Tanimoto M, McKenzie S, et al. Leukemia 3:440–445, 1989.) which reacts with 60–70% of samples of blasts from patients with ANLL. M195 also binds to early myeloid cells (CFU-GM) and some monocytes but not to the earliest myeloid progenitors. The target antigen is not expressed on any other hematopoietic or non-hematopoietic tissue. Antibodies to a related antigen on the same protein (CD33), My9 and L4F3, are currently being used to purge bone marrow of ANLL before autologous transfusion (Bernstein I D, Singer J W, Andrews R G, et al. J Clin Invest 79:1153–1159, (1987); Griffin J D, Linch D, Sabbath K, et al. Leukemia Res 8:521–534, 1984.). M195 is rapidly internalized into cells after binding and this effect can enhance delivery of radiometals, radioiodine or conjugated toxins into cells (Divgi C R, Minniti J G, Old L J, Scheinberg D A Amer Assoc Cancer Res 30: Abs #1606, 1989). M195 is able to kill leukemia cells with rabbit or guinea pig complement, but not by use of human complement or human antibody-dependent cellular cytotoxicity in vitro. Activation of these mediators in vitro has correlated with these effects in vivo (Houghton A N, Mintzer D, Cordon-Cardo C, et al. Proc Natl Acad Sci USA 82:1242–1246, (1985)), but it is not known if the lack of in vitro effects will predict lack of in vivo effects. Because M195 also reacts with early myeloid cells, normal marrow progenitors may be affected also.

Studies of M195 in Humans. In order to determine the toxicity, biodistribution, pharmacology and dosimetry of radiolabeled M195 in humans, a pilot trial involving 9 patients with ANLL was initiated under IRB approval (#89-113A(1)) at Memorial Hospital. The trial involves a dose escalation of M195 (1, 5, 25 mg/m^2 per dose) with 4 doses of antibody given over 1 week. In this trial the first (and sometimes second) doses of antibody were trace-labeled with iodine-131 to allow whole body imaging for biodistribution and half-life calculations, and accurate pharmacology and dosimetry studies based on repeated blood and marrow samplings.

The following data were derived from the first three patients treated:

Feature Studied	Patient #1	Patient #2	Patient #3
Age/Sex/Dx	25 y.o. male-AML, refractory	20 y.o. woman-AML, relapsed	24 y.o. male AML, relapsed
Prior Therapy	Multiple chemo-therapies	Multiple chemo-therapies allogeneic BM transplant	Multiple chemo-therapies
Circulating blasts	30,00/mm^2	100/mm^2	10/mm^2
BM Status	Packed	Hypoplastic	Partially Hypoplastic
Clinical Status	Severely ill; multiple infections; bleeding	Stable	Stable
Doses	1.5 mg x4	1.5 mg x4	2.0 mg x4
Toxicity	Transient itching during infusions	None	None
T ½ Blood Images	<1 day All areas of leukemia including predominantly BM; no blood pool (18–89 hours)	>4 days Blood Pool	3 days Marrow; No Blood Pool
M195 IgG per leukemia cell (Estimated)	1200	4000	1400

We concluded that there was rapid and specific uptake of M195 onto target cells and that sufficient numbers of M195 were bound to each cell to allow a therapeutic dose of radioisotope, even at this first dose level (1 mg/m^2), at wide variations of tumor burden (patient 1: >1 trillion cells; patient 2: <100 billion cells).

Microdosimetry of I–123. The localization of the M195 mAb on the surface of the leukemia cell and its subsequent incorporation into the cytoplasm presents an opportunity to select a radionuclide with properties that exploit this situation. Electron emissions that have a range on the order of a cell diameter (10–20 microns) deposit a large fraction of their energy within the cell (Kassis A I, Adelstein S J, Haycock C, Sastry K S R. Radiation Research 84:407–425 (1980)). Electron capture (EC) is a mode of decay that gives rise to a large percentage of such low energy emissions. The isotopes of iodine that decay by EC include I-125 (T-½=60 d) and I-123 (T-½=14 h), the latter being advantageous in terms of a physical half-life matching the expected biological clearance times. From a microdosimetric point of view, in cells on the order of 10–12μ in diameter, it is more effective than I-131. Another advantage of I-123 is relatively low marrow dose per millicurie from the 127 key (13.7%) conversion electron. The isotope also has superior imaging characteristics for confirming dose distribution as well as the dosimetry.

Assuming that at most half of the labeled dose is concentrated uniformly in the marrow, and T-effective=T-physical, the dose to the marrow cavity is 0.27 rad/mCi administered. Thus, only 27 rad/100 mCi would be administered. Examination of radiation exposure of nursing staff, radiation dose to other organs within the patient and the cost of the radionuclide, activities of about 200 mCi would appear to be safe and practical. However, with the above assumptions, 200 mCi would be sufficient to kill only about 10^{10} leukemia cells. This is based on the following arguments:

200 mCi represents 5.4×10^{14} atoms of I-123. Based on presented and published work of Howell, Sastry, Rao et al (Howell, private communication) it appears that approximately 1 MeV of energy must be deposited in the nucleus on average from such electron capture nuclides to achieve cell kill. This corresponds to approximately 2000 disintegrations

from atoms in the cytoplasm (Kassis A I, Adelstein S J, Haycock C, Sastry K S R. Radiation Research 84:407–425 (1980)), or in this case, also atoms on the cell surface since the radius of the cell may only be about 1–2 microns greater than that of the nucleus. Based on observed biological clearance in initial patient studies here and the known physical half life (14 hours) of I-123 a reasonable assumption is that about half the atoms taken up by the cell will decay while on or in the cell. (There are approximately 10,000 surface binding sites per cell. Labelling carried out at 1 atom of I-123 per 8 antibodies will result in 200 mCi of I-123 on about 1.0 mg of antibody.) Ideally 200 mCi (5.4×10^{14} atoms) at 5000 atoms of I-123 disintegrating per cell should kill 1.1×10^{11} cells. However, since the targeting efficiency is only about 20–30%, 200 mCi will theoretically only kill about 3×10^{10} cells at most.

Pharmacologic limits and need for cytoreduction. Based on the first phase I study of M195 and on achievable and practical levels of ^{123}I-M195 specific activities, described in 2.5 (determined by physical properties of both antibody and isotope, data on antigen expression and antibody immunoreactivity, radiation safety concerns and costs of isotope), it does not seem practical to expect at this time that the ^{123}I-M195 will effectively target to 12 logs of cells (typical tumor burden). Therefore, we will cytoreduce the patients 3 days before M195 therapy with a small dose of Ara-C calculated to kill 1–2 logs of leukemia cells and leave 10^{10}–10^{11} cells at day 4 when ^{123}I-M195 begins. This dose of Ara-C will be in no way therapeutic and will therefore still enable us to determine the therapeutic effects of M195. The toxicity of this chemotherapy should also be minimal.

Radiometal Chelate-Conjugated Antibodies. Radiometals have been attached to monoclonal antibodies via bifunctional chelates for diagnostic imaging and therapy. We have proposed the use of alpha particle emitting nuclides, Lead 212 or Bismuth 212 as the optimal radiotherapeutic nuclides for use in killing leukemia cells based on emission characteristics and pharmacology studies in animals (Brechbiel M W, Gansow O A, Atcher R W, et al. Inorg Chem 25:2772–2781, 1986; Kumar K, Magerstadt M, Gansow O A J Chem Soc Chem Commun, 3:145–146 (1989)). Rotationally hindered benzyl derivates of DTPA and planar cage structures such as DOTA have been developed recently to chelate bismuth (Gansow O A, Atcher R W, Link D C, Friedman A M, Sever R H, Anderson W, Scheinberg D A, Strand M In: F F Knapp and J A Butler (eds) Radionuclide Generators, Vol 241, pp 215–230. Washington, DC. ACS. (1984)). These chelands have been conjugated to M195 without loss of immunoreactivity and the chelated Bismuth 212 can be made without loss of binding activity. These chelates are able to kill leukemia cells in model systems in vitro. Animals models of other Bismuth conjugated antibodies show no loss of bismuth to non-target organs.

FIG. 11: Schematic diagram of the distribution of the M195 antigen in human tissues. The antigen is not known to be present on any adult non-hematopoietic tissues, so they are not shown. The distribution in the hematopoietic cells is shown.

FIG. 12: Posterior and anterior whole body gamma camera images of patient #1, injected 18 hours earlier with 5 mCi ^{131}I M195 (1.5 mg). All known areas of leukemic involvement (bone marrow, spleen, liver, mediastinal chloroma) show marked uptake of M195.

EXPERIMENT 5

(Lemoli Abstract)

Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia (AML): In Vitro Treatment with Myeloid-Specific Monoclonal Antibodies (MoAbs) and Drugs in Combination

We report the results of a preclinical study comparing four different purging protocols using promyelocytic human cell line HL-60 and myeloid leukemic progenitor cells [CFU-GM-(L)] from AML patients assayed in semisolid culture. We studied the antileukemic effect of 1] complement-dependent lysis by 2 different MoAbs (M195 and F23; 40 ug/ml), reactive with distinct antigens found on early myeloid cells and monocytes, used alone and in combinations; 2] 4-Hydroperoxycyclophosphamide (4-HC) (100 uM/L) alone or 3] combined with VP-16 (5 ug/ml) and 4] a cocktail of 1–3 protocols as above (ie combined immunochemotherapy). Four logs of HL-60 tumor cell elimination was observed after 1 hour incubation with both MoAbs plus 4-HC+VP-16 while the single treatment (ie immunotherapy or chemotherapy) provided 1.5 and 3.0 logs of colony forming inhibition respectively.

When the same protocols were tested on fresh leukemic cells from 4 patients with AML we observed a mean value of CFU-GM-(L) inhibition of 94.7%, 95.5% and 98.6% after MoAbs and complement lysis, 4-HC and 4-HC+VP-16 treatment respectively. The combined treatment of MoAbs and 4-HC+VP-16 produced a 3-log reduction of CFU-GM-(L) colony formation. By comparison the mean recovery of committed normal bone marrow progenitors after incubation with MoAbs and complement was 19.5% for CFU-GM, 32.3% for BFU-E and 16.7% for CFU-MIX. 4-HC+VP-16 treatment showed 4.4% CFU-GM and 5.6% BFU-E recovery. Preliminary data obtained on highly purified CD34+ blast cells, enriched by positive selection, demonstrated the capacity of the immunochemotherapy combined protocol to spare the earliest hematopoietic colony-forming cells. In summary, our results indicate that immunochemotherapy may be a more effective purging strategy for autologous bone marrow transplantation in patients with acute myelogenous leukemia.

EXPERIMENT 6: PREPARATION OF PATIENT FOR REINFUSION

(Reference to Experiment 3) (Gulati Protocol)

Patients receiving autologous bone marrow transplantation for the treatment of acute myelogenous leukemia still largely fall due to relapse. This may be due to inadequate preparation of the host or due to inadequate purging. We propose to attempt to improve both aspects of this regimen using cytotxic monoclonal antibodies specific for acute myelogenous leukemia cells and early myeloid progenitors. In htis trial, we will first assess the safety and efficacy of ^{131}I-M195 to improve on patient conditioning. If this is successful, in a later trial, we will add M195 purging of bone marrow as well. We will not try both at once, at this time, in order that we might assess the effects of each alone.

Phase I toxicity and pharmacology studies with ^{131}I-M195 done here suggest that in patients with low tumor burden 3–5 rads per mCi will be delivered to the marrow with ^{131}I-M195. Thus, in the proposed trial here, we expect to deliver specifically to the marrow up to an additional 200, 400 and 600 rads at the three escalating dose levels of 131-M195. Doses to other organs should not be significant.

M195 targets to bone marrow and carries a long range isotope for the preparative regimen such as ^{131}I or ^{90}Y instead of the short range isotope such as Bismuth or auger electron generators that are used for killing leukemias as described in Experiment 3 (above) to kill not only the cell on which it is attached but also nearby normal and neoplastic

cells. This allows reinfusion of the new marrow. At the same time it kills residual neoplastic cells that may escape the chemotherapy or radiotherapy given as part of the conventional regimen.

This preparative regimen may be (is)? useful for all allogeneic transplants, even those for non-hematopoietic cancer. It may be? is useful in autologous transplants as well.

EXPERIMENT 7: GENETIC RETROVIRAL VECTOR SECTION

Method for Antibody Targeting of Genetic Information into Hematopoietic Cells

Retroviral vectors may be used to introduce exogenous DNA sequences into hematopoietic progenitors and pluripotent stem cells. (Claudio Bordignon, et al, PNAS vol 86:6748–6752 (1989); Stefan Karlsson, et al., PNAS vol 85:6062–6066 (1988); Bruno Goud, Virology vol 163:251–254 (1988); E. Gilboa, Bioessays vol 5(6):252 (1986); W F Anderson, et al, Basic Life Sci. vol 37:59 (1986)) Genetic information encoding, for example, a new gene or part of a gene required for enzyme or hemoglobin function or another required structural protein, is attached to M195 by a retroviral vector. Retroviral vectors usually require receptors for entry into target cells. M195 will substitute for the usual envelope protein involved in this entry and will thus confer specificity for the appropriate cells.

M195 can be attached to the outside of the vector by chemical or genetic means: the M195 could be directly crosslinked to the viral envelope proteins; the M195 could be bound to another antibody or fragment which is directed against the viral envelope proteins; protein A can be inserted genetically into the envelope to bind M195; or M195 can be inserted genetically into the envelope. (see FIG. 13).

REFERENCES

(Ref.a)

1. Civin C. I., Mirro J., Banquerigo M. L. MY-1, A New Myeloid-Specific Antigen Identified by a Mouse Monoclonal Antibody. Blood 57:842–845 (1981).
2. Griffen J. D., Ritz J., Nadler L. M., Schlossman S. F. Expression of Myeloid Differentiation Antigens on Normal and Malignant Myeloid Cells. J. Clin. Invest 68:932–941 (1981).
3. Perussia B., Trichieri G., Lebman D., Jankiewicz J., Lange B., Rovera G. Monoclonal Antibodies that Detect Differentiation Surface Antigens on Human Myelomonocytic Cells. Blood 59:382–392 (1982).
4. Andrews R. G., Torok-Storb B., Bernstein I. D. Myeloid-associated Differentiation Antigens on Stem Cells and Their Progeny Identified by Monoclonal Antibodies. Blood 62:124–132 (1983).
5. Griffen J. D., Linch D., Sabbath K., Larcom P., Schlossman S. F. A Monoclnoal Antibody Reactive with Normal and Leukemic Human Myeloid Progenitor Cells. Leuk Res 8:521–534 (1984).
6. Civin C. I., Strauss L. C., Brovall C., Fackler M. J., Schwartz J. F., Shaper J. H. Antigenic Analysis of Hematopoiesis. IIIA Hematopoietic Progenitor Cell Surface Antigen Defined by a Monoclonal Antibody Raised Against KG1a Cells. J Immunol 133L157–165 (1984).
7. Katz F. E., Tindle R., Sutherland D. R., Greaves M. F. Identification of a Membrane Glycoprotein Associated with Hematopoietic Progenitor Cells. Leuk Res 9:191–198 (1985).
8. Askew D. S., Eaves A. C., Takei F. NHL-30.5: A Monoclonal Antibody Reactive with an Acute Myeloid Leukemia (AML)-associated Antigen. Leuk Res 9:135–145 (1985).
9. Ferrero D., Gab bianelli M., Peschle C., Lange B., Rovera G. Surface Phenotypes of Human Hematopoietic Progenitor Cells Defined by Monoclonal Antibodies. Blood 66:945–502 (1985).
10. Drexler H. G., Sagawa K., Menon M., Minowada J. Reactivity Patterns of Myeloid Monoclonal Antibodies with Emphasis on MCS-2. Leuk Res 10:17–23 (1986).
11. Peng R., Al-Katib A., Knowles D. M., Lu L., Borxmeyer H., Tolidjiian B., Chiao J-W, Koziner B., Wang C. Y. Preparation and Characterization of Monoclonal ANtibodies Recognizing Two Distinct Differentiation Antigens (Pro-Im1, Pro-Im2) on Early Hematopoietic Cells. Blood 641169–1178 (1984).
12. Wisniewski D., Knowles R., Wachter M., Strife A., Clarkson B. Expression of Two Natural Killer Cell Antigens, H-25 and H-366. by Human Immature Myeloid Cells and by Erythroid and Granulocytic/Monocytic Colony-Forming Units. Blood 69:419–429 (1987).
13. Strife A., Lambek C., Wisniewski D., Gulati S., Gasson J. C., Golde D. W., Welte K., Gabrilove J. L., Clarkson B. Activities of Four Purified Growth Factors on Highly Enriched Human Hematopoietic Progenitor Cells. Blood 69:1508–1523 (1987).
14. Ball E. D., Mills L. E., Coughlin C. T., Beck R., Cornwell G. G. Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia: In Vitro Treatment with Myeloid Cell-Specific Monoclonal Antibodies. Blood 68:1311–1315 (1986).
15. Ball E. D., bernier G. M., Cornwell G. G., McIntyre O. R., O'Donnel J. F., Fanger M. W. Monoclonal Antibodies to Myeloid Differentiation Antigens: In Vivo Studies of Three Patients with Acute Myelogenous Leukemia. Blood 62:1203–1210 (1983).
16. Tanimoto M., Scheinberg D. A., Cardo C. C., Huie D., Clarkson B. D., Old L. J. Restricted Expression of an Early Myeloid and Monocytic Cell Surface Antigen Defined by Monoclonal Antibody M195. Leukemia 3:339–348 (1989).
17. Andrews R. G., Takahashi M., Segal G. M., Powell J. S., Bernstein I. D., Singer J. W. The L4F3 Antigen Is Expressed by Unipotent and Multipotent Colony-forming Cells But Not by Their Precursors. Blood 68:1030–1035 (1986).
18. Bernstein I. D., Singer J. W., Andrews R. G., Keating A., Powell J. S., Bjornson B. H., Cuttner J., Najfeld V., Reaman G., Raskind W., Sutton D. M. C., Fialkow P. J. Treatment of Acute Myeloid Leukemia Cells In Vitro with a Monoclonal ANtibody Recognizing a Myeloid Differentiation Antigen Allows Normal Progenitor Cells to be Expressed. J Clin Invest 79:1153–1159 (1987).
19. Bennett J. M., Catovsky D., Daniel M. T., Flandrin G., Galton D. A. G., Gralnick H., Sultan C. Proposed Revised Criteria for the Classification of Acute Myeloid Leukemia. Ann Intern Med 103:626–629 (1985).
20. Peiper S. C., Lemons R. S., Ashmun R. A., Look A. T. DNA-Mediated Transfer of the Gene Encoding p67 (DCD33), a Myeloid Differentiation Antigen Recognized by the MY9, L1B2, and L4F3 Monoclonal Antibodies. In: mcMichael A. J., ed. Leukocyte Typing III. Oxford: Oxford University Press, 622–625 (1986).
21. Alvey P. L., Greaves M. F. A Computer Program for Interpreting Immunophenotypic Data as an Aid to the Diagnosis of Leukemia. Leukemia 1:527–540 (1987).
22. Neame P. B., Soamboonsrup P., Browman G. P., Meyer R. M., Bender A., Wilson W. E. C., Walker I. R, Saeed N., McBride J. A. Classifying Acute Leukemia by Immunophenotyping: a COmbined FAV-Immunologic Classification of AML. Blood 68:1355–1362 (1986).

23. Pessano S., Palumbo A., Ferrero D., Pagliardi G. L., Bottero L., Lai S. K., Meo P., Carter C., Hubbell H., Lange B., Rovera G. Subpopulation Heterogeniety in Human Acute Myeloid Leukemia Determined by Monoclonal Antibodies. Blood 64:275–281 (1984).
24. Griffin J. D., Mayer R. J., Weinstein H. J., Rosenthal D. S., Coral F. S., Bevendga R. P., Schlossman S. f. Surface Marker Analysis of Acute Myeloblastic Leukemia: Identification of Differentiation-Associated Phenotypes. Blood 62:557–563.
25. Van der Reijden H. J., Van Rhenen D. J., Lansdorp P. M., Van't Veer M. D., Langenhuijsen M. M. A. C., Engelfriet C. P., Von dem Borne A. F. G. K. A Comparison of Surface Marker Analysis and FAV Classification in Acute Myeloid Leukemia. Blood 61:443–448 (1983).
26. Linch D. C., Allen C., Beverley P. C. L., Bynoe A. G., Scott C. S., Hogg N. Monoclonal Antibodies Differentiating Between Monocytic and Nonmonocytic Variants of AML. Blood 63:566–573 (1984).

REFERENCES

(Ref.b)

1. Foon K. A., Todd R. F. Immunologic Classification of Leukemia and Lymphoma. Blood 68:1–31 (1986).
2. Alvey P. L., Greaves M. F. A Computer Program for Interpreting Immunophenotypic Data as an Aid to the Diagnosis of Leukemia. 1:527–540 (1987).
3. Wilson W. E. C., Walker I. R., Saeed N., McBride J. A. Classifying Acute Leukemia by Immunophenotyping. A COmbined FAB-Immunologic Classification of AML. Blood 68:1355–1362 (1986).
4. Griffin J. D., Linch D., Sabbath F., Larcam P., Schlossman S. F. A Monoclonal Antibody Reactive with Normal and Leukemic Human Myeloid Progenitor Cells. Leuk Res 8:521–534 (1984).
5. Dinndorf P. A., Andrews R. G., Benjamin D., Ridgway D., Wolff L., Bernstein I. D. Expression of Normal Myeloid-Associated ANtigens by Acute Leukemia Cells. Blood 67:1048–1053 (1986).
6. Pessano S. Palumbo A. Ferrero D., Pagliardi G. L., Bottero L., Lai S. k., Meo P., Carter C., Hubbell H., Lange B., Rovera G. Subpopulation Heterogeneity in Human Acute Myeloid Leukemia Determined by Monoclonal Antibodies. Blood 64:275–281 (1984).
7. Griffin J. D., Mayer R. J., Weinstein H. J., Rosenthal D. S., Coral F. S., Bevendga R. P., Schlossman S. F. Surface Marker Analysis of Acute Myeloblastic Leukemia: Identification of Differentiation-Associated Phenotypes. Blood 62:557–563 (1983).
8. Van der Reijden H. J., Van Rhenen D. J., Lansdorp P. M., Van't Veer M. D., Langenhuijsen M. M. A. C., Engelfriet C. P., Von dem Borne A. F. G. K. A Comparison of Surface Marker Analysis and FAV Classification in Acute Myeloid Leukemia. Blood 61:443–448 (1983).
9. Linch D. C., ALlen C., Beverley P. C. L., Bynoe A. G., Scott C. S., Hogg N. Monoclonal Antibodies Differentiation Between Monocytic and Nonmonocytic Variants of AML. Blood 63:566–573 (1984).
10. Drexler H. G., Minowada J. The Use of Monoclonal Antibodies for the Identifcation and Classification of Acute Myeloid Leukemias. Leuk Res 10:279–290 (1986).
11. Ball E. D., Bernier G. M., Cornwell G. G., McIntyre O. R., O'Donnel J. F., Fanger M. W. Monoclonal Antibodies to Myeloid Differentiation Antigens: In Vivo Studies of Three Patients with Acute Myelogenous Leukemia. Blood 62:1203–1210 (1983).
12. Ball E. D., Mills L. E., Coughlin C. T., Beck R., Cornwell G. G. Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia: In Vitro Treatment with Myeloid Cell-Specific Monoclonal Antibodies. Blood 68:1311–1315 (1986).
13. Griffin J. D., Lowenberg B. Clonogenic Cells in Acute Myeloblastic Leukemia. Blood 68:1185–1195 (1986).
14. Lange B., Ferrero D., Pessano S., Palumbo A., Faust J., Meo P., Rovero G. Surface Phenotype of Clonogenic Cells in Acute Myeloid Leukemia Defined by Monoclonal ANtibodies. Blood 64:693–700 (1984).
15. Sabbath K. D., Ball E. D., Larcom P., Davis R. B., Griffin J. D. Heterogeneity of Clonogenic Cells in Acute Myeloblastic Leukemia. J Clin Invest 75:746–753 (1985).
16. Lowenberg B., Bauman J. G. J. Further Results in Understanding the Subpopulation Structure of AML: Clonogenic Cells and Their Progeny Identified by Differentiation Markers. Blood 66:1225–1232 (1985).
17. Peiper S. C., Lemons R. S., Ashmun R. A., Look A. T. DNA-Mediated Transfer of the Gene Encoding p67 (CD33), a Myeloid Differentiation ANtigen Recognized by the MY9, L1B2, and L4F3 Monoclonal ANtibody. In: McMichael A J, ed. Leucocyte Typing III. New York: Oxford University PRess, 622–625 (1987).
18. Berenson R. J., Bensinger W. L., Kalamasz D. Positive Selection of Viable Cell Populations Using Avidin-Biotin Immunoadsorption. J Immunol Methods 91:11–19 (1986).
19. Katz F. E., Tindle R., Sutherland D. R., Geraves M. F. Identificaiton of a Membrane Glycoprotein Associated with Hematopoietic PRogenitor Cells. Leuk Res 91:191–198 (1985).
20. Civin C. L., Strauss L. C., Brovall C., Fackler M. J., Schwartz J. F., Shaper J. H. Antigenic Analysis of Hematopoiesis. III. A Hematopoietic Progenitor Cell Surface ANtigen Defined by a Monoclonal Antibody Raised Against KG1a Cells. J Immunol 133:157–165 (1984).
21. Strauss L. C., Rowley S. D., LaRussa V. F., Sharkis S. J., Stuart R. K., Civin C. I. Antigenic Analysis of Hematopoiesis. V. Characterization of My-10 Antigen Expression by Normal Lymphohematopoietic Progenitor Cells. Exp Hematol 14:878–886 (1986).
22. Askew D. S., Eaves A. C., Eaves C. J., Takei F. Restricted Expression of a New Acute Myelogenous Leukemia-Associated Antigen (NHL-30.5) on Normal Hemopoietic Progenitor Cells. Blood 67:2098–1102 (1986).
23. Askew D. S., Eaves A. C., Takei F. NHL-30.5: A Monoclonal Antibody Reactive with an Acute Myeloid Leukemia (AML)-Associated Antigen. Leuk Res 9:135–145 (1985).
24. Bernstein J. D., Singer J. W., Andrews R. G., Keeting A., Powell J. S., Bjornson B. H., Cuttner J., Najfeld V., Reaman G., Raskin W., Sutton D. M. C. Fialkow P. J. Treatment of Acute Myeloid Leukemia Cells In Vitro with Monoclonal Antibody Recognizing a Myeloid Differentiation Antigen Allows Normal Progenitor Cells to be Expressed. J Clin Invest 79:1153–1159 (1987).
25. Andrews R. G., Takahashi M., Segal G. M., Powell J. S., Bernstein I. D., Singer J. W., The L4F3 Antigen Is Expressed By Unipotent and Multipotent Colony-forming Cells But Not By Their Precursors, Blood 68:1030–1035 (1986).
26. Andrews R. G., Torok-Storb B., Bernstein I. D., Myeloid-associated Differentiation Antigens on Stem Cells and Their Progeny Identified by Monoclonal Antibodies. Blood 62: 124–132 (1983).

27. Griffin J. D., Ritz J., Nadler L. M., Schlossman S. F., Expression of Myeloid Differentiation Antigens on Normal and Malignant Myeloid Cells. J. Clin. Invest 68:932–941 (1981).

28. Peng R., Al-Katib A., Knowles D. M., Lu L., Broxmeyer H., Tolidjian B., Chiao J-W, Koziner B., Wang C. Y., Preparation and Characterization of Monoclonal Antibodies (Pro-Im1, Pro-Im2) on Early Hematopoietic Cells. Blood 64:1169–1178 (198).

29. Wisniewski D., Knowles R., Wachter M., Strife A., Clarkson B., Expression of Two Natural Killer Cell Antigens, H-25 and H-366, By Human Immature Myeloid Cells and By Erythroid and Granulocytic/Monocytic Colony-Forming Units. Blood 69:419–429 (1987).

30. Scheinberg D. A., Tanimoto M., McKenzie S., Strife A., Old L. J., Clarkson B. D., Monoclonal antibody M195: A Diagnostic Marker for Acute Myelogenous Leukemia. Leukemia (1989) (in press).

31. Cairncross J. C., Mattes M. J., Beresford H. R., Albino A. P., Houghton A. N., Lloyd K. O., Old L. J., Cell surface Antigens of Human Astrocytoma Defined by Mouse Monoclonal Antibodies: Identification of Astrocytoma Subsets. Proc. Natl. Acad. Sci. USA 79:5641–5645 (1982).

32. Shiku H. Takahashi T., Oettgen H. F. Old L. J., Cell Surface Antigens of Human Maliganant. II. Serological Typing With Immune Adherence Assays and Definition of Two New Surface Antigens. J Exp Med 144:873–881 (1976).

33. Mattes J. M., Tanimoto M., Pollack M. S., Maurer D. H., Preparing Monolayers of Non-adherent Mammalian Cells. J. Immunol Methods 61:145–150 (1983).

34. Cayre Y., Raynal M. C., Darzykiewicz Z., Dorner M. H., Model For Intermediate Steps in Monocytic Differentiation. Proc. Natl. Acad. Sci. USA 84:7619–7623 (1987).

35. Welt S., Carswell E. A., Vogel C. W., Oettgen H F, Old L. J., Immune and Non-immune Effector Function of IgG3 Mouse Monoclonal Antibody R24 Detecting the Disialoganglioside GD3 On the Surface of Melamoma Cells. Clin Immunol Immunopathol 45:214–229 (1987).

36. Old L. J., Stockert E., Boyse E. A., Kim J. H., Antigenic modulation: Loss of TL Antigen From Cells Exposed to TL Antibody. Study of the Phenomenon In Vitro. J. Exp. Med. 127:523–539 (1968).

37. Berkowitz R. S., Umpierre S. A., Goldstein D. P., Andersen D. J., Cross Reactivity of Monoclonal Antibodies Directed Against Lymphocyte Markers with Trophoblast Cells of Normal Placenta, Hydatidiform Mole, and Gestational Choriocarcinoma. Gynecol Oncol 29:94–100 (1988).

38. Sakamoto J., Furukawa K., Cordon-Cardo C., Yin B. W. T., Rettig W. J., Oettgen H. F., Old L. J., Lloyd K. O. Expression of Lewis (b), X and Y Blood Group Antigens in Human Colonic Tumors and Normal Tissue and in Human Tumor-Derived Cell Lines. Cancer Res. 46:1553–1561 (1986).

39. Drexler H. G., Sagawa K., Menon M., Minowada J., Reactivity Patterns of Myeloid Monoclonal Antibodies with Emphasis On MCS 2, Leukemia Res 10:17–23 (1986).

40. McMichael A. J., ed. Leukocyte Typing III. New York: Oxford University Press, 577–602 (1987).

41. Krolick K. A., Villenmez C., Isakson P., Uhr J. W., Vitetta E. S., Selective Killing of Normal or Neoplastic B Cells by Antibodies Coupled to the A Chain Ricin. Proc. Natl. Acad. Sci. USA 77:5419–5423 (1980).

42. Shimazaki C., Wisniewski D., Scheinberg D. A., Atpodien J., Strife A., Gulati S., Fried J. Wisniewolski R., Wang C. Y., Clarkson B. D., Elimination of Myeloma Cells From Bone Marrow Using Monoclonal Antibodies and Magnetic Immunobeads. Blood 72:1248–1254 (1988).

REFERENCES

(Ref.c)

1. Kohler G., Milstein C. Continuous Culture of Fused cells Secreting Antibodis of Pre-defined Specificity. Nature 256:495–497 (1975).

2. Larson S. M. Radiolabeled Monoclonal Anti-tumor Antibodies in Diagnosis and Therapy. J. Nucl. Med 26:538–545 (1985).

3. Houghton A. N., Scheinberg D. A. Monoclonal Antibodies: Potential Applications to the Treatment of Cancer. Sem Oncol 13:165–179 (1986).

Goldenberg D. M., Deland F., Kim E. et al. Use of Radiolabeled Antibodies to Carcinoembryonic Antigen for the Detection and Localization of Diverse Cancers by External Photoscanning. N. Engl. J. Med. 298:1384–1388 (1978).

5. Bunn P. A., Jr., Carrasquillo J. A., Keenan A. M. et al. Imaging of T-cell Lymphoma by Radiolabeled Monoclonal Antibody. Lancet 2:1219–1221 (1984).

Larson S. M., Brown J. P., Wright P. W. et al. Imaging of Melanoma With 1–131 Labeled Monoclonal Antibody. J. Nucl. Med 24:123–129 (1983).

7. Press O. W., Eary J. F., Badger C. C. et al. Treatment of Refractory Non-hodgkin's Lymphoma With Radiolabeled MB-1 (Anti-CD37) Antibody. J. Clin. Oncol 7:1027–1038 (1989).

8. Rosen S., Zimmer A., Golman-Leikin R. et al. Radioimmunodetection and Radioimmunotherapy of Cutaneous T-cell Lymphomas Using an ^{131}I-labeled Monoclonal Antibody. J. Clin. Oncol. 5:562–573 (1987).

9. Carrasquillo J. A., Bunn P. A., Keenan A. A. et al. Radioimmunodetection of Cutaneous T-cell lymphoma with ^{111}In-labeled T101 Monoclonal Antibody. N. Engl. J. Med. 315:673–680 (1986).

10. Eptein A. L., Zimmer A. M., Spies S. M. et al. Radioimmunodetection of Human B-cell Lymphomas With a Radiolabeled Tumor-specific Monoclonal Antibody (Lym-1). In Maliganant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic advances, Cavalli F., Bonadonna G., Rozencweig M., eds Boston Martinus Nijhoff (1985)

11. DeNardo S. J., DeNardo G. L., O'Grady L. F. et al. Pilot Studies of Radioimmunotherapy of B Cell Lymphoma and Leukemia Using I-131 Lym-1 monoclonal Antibody. Antibody Immunoconj Radiopharm 1:17–33 (1988).

12. Rao D. V., Narra V. R., Howell R. W. et al. In-vivo Radiotoxicity of DNA-incorporated ^{125}I Compared With That of Densely Ionizing Alpha-particles. Lancet vol.2:650–653 (Sep. 16, 1989).

13. Woo D. V., Li D., Mattis J. A. et al. Cancer Res. 49:2952–2952 (1989).

14. Scheinberg D. A., Strand M. Kinetic and Catabolic Considerations of Monoclonal Antibody Targeting in Erythroleukemic Mice. Cancer Res. 43:265–272 (1983).

15. Khaw B. A., Cooney J., Edgington T., et al. Differences in Experimental Tumor Localization of a Dual-labeled Monoclonal Antibody. J. Nucl. Med. 27:1293–1299 (1986).

16. Carrasquillo J. A., Mulshine J. L., Bunn P. A., Jr. et al. Indium-111 T101 Monoclonal Antibody is Superior to Iodine-131 T101 in Imaging of Cutaneous T-cell Lymphoma. J. Nucl. Med. 28:281–287 (1987).

17. Anderson W. M., Strand M., Radiolabeled Antibody: Iodine Versus Radiometal Chelates. NCI Monogr 3:149–151.

18. Lamm M. E., Boyse E. A., Old L. J. et al. Modulation of TL (thymus-leukemia) Antigens by Fab-fragments of TL Antibody. J. Immunol. 101:99–103 (1968)

19. Shawler D. L., Micelli M. C., Wormsley S. B., Royston L., Dillman R. O. Induction of In Vitro and In Vivo Antigenic Modulation by the Anti-human T-cell Monoclonal Antibody T101. Cancer Res. 44:5921–5927 (1984).

20. Press O. W., Farr A. G., Borroz K. L et al. Endocytosis and Degradation of Monoclonal Antibodies Trargeting Human B-cell Malignancies. Cancer Res. 49:4906–49012 (1989).

21. Matzku S., Brocker E.-B., Brugen J. et al. Modes of Binding and Internalization of Monoclonal Antibodies to Human Melanoma Cells Lines. Cancer Res. 46:3848–3854 (1986).

22. Wang B. So, Lumanglas A. L., Silva J. et al. Internalization and Re-expression of Antigens of Human Melanoma Cells Following Exposure to Monoclonal Antibody. Cell Immunol. 106:12–21 (1987).

23. Olsnes S., Sandvig K., Petersen O. W., van Deurs B. Immunotoxins—Entry Into Cells and Mechanisms of Action. Immunol. Today 10:290–295 (1989).

24. Tanimoto M., Scheinberg D. A., Cordon-Cardo C., Huie D., Clarkson B. D., Old L. J., Restricted Expression of an Early Myeloid and Monocytic Cell Surface Antigen Defined by Monoclonal Antibody M195. Leukemia 3:339–348 (1989).

25. Scheinberg D. A., Tanimoto M., McKenzie S., Strife A., Old L. J., Clarkson B. D., Monoclonal Antibody M195: A diagnostic Marker for Acute Myelogenous Leukemia. Leukemia 3:440–445 (1989).

26. Mittler R. S., Talle M. A., Carpender K. et al. Generation and Characterization of Monoclonal Antibodies Reactive With Human B Lymphocytes. J. Immuno 131:1754–1761 (1983).

27. Nemerow G. R., Wolfert R., McNaughton M. E. et al. Identification and Characterization of the Epstein-Barr Virus Receptor on Human B Lymphocytes and Its Relationship to the C3d Complement Receptor (CR2). J. Virol. 55:347–351 (1985).

28. Hunter W. M., Greenwood F. C., Preparation of Iodine-131 Labelled Human Growth Hormone of High Specificity Activity. Nature 194:495–496 (1962).

29. Lindmo T., Boven E., Cuttitta F. et al. Determination of Immunoreactive Fraction of Radiolabeled Monoclonal Antibody By Linear Extrapolation to Binding at Infinite Antigen Excess. J. Immunol Methods 72:77–89 (1984).

30. Badger C. C., Krohn K. A., Bernstein I. D. In Vitro Measurement Of Avidity of Radioiodinated Antibodies. Nucl. Med. Biol. 14:605–610 (1987).

Krejcarek G. E., Tucker K. L. Covalent Attachment of Chelating Groups to Macromolecules. Biochem. Biophy. Res. Commun 77:581–585 (1977)

32. The Gansow Paper in Cancer Res. 89.

What is claimed is:

1. A therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto, wherein the cytotoxic agent is a polypeptide toxin.

2. A therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto, wherein the cytotoxic agent is an alpha particle emitter.

3. The therapeutic agent of claim 2, wherein the alpha particle emitter is selected from the group consisting of Lead-212, Bismuth-212, and Astatine-212.

4. The therapeutic agent of claim 3, wherein the alpha particle is Bismuth-212.

5. The therapeutic agent of claim 3, wherein the alpha particle emitter is conjugated to the monoclonal antibody by means of a bifunctional chelate.

6. A therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto, wherein the cytotoxic agent is a beta particle emitter selected from the group consisting of Scandium-47, Rhenium-186, Rhenium-188, and Yttrium-90.

7. The therapeutic agent of claim 6, wherein the beta particle emitter is Scandium-47.

8. The therapeutic agent of claim 6, wherein the beta particle emitter is Yttrium-90.

9. A therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto, wherein the cytotoxic agent is an auger electron generator selected from the group consisting of Iodine-123, Bromine-77, and Indium-111.

10. The therapeutic agent of claim 9, wherein the auger electron generator is Iodine-123.

11. A therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto, wherein the cytotoxic agent is a fissionable nuclide selected from the group consisting of Boron-10 and an Actinide.

12. A method of treating acute or chronic myeloid leukemia in a human patient which comprises administering to the patient an amount of a therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto sufficient to bind to, and be internalized by, leukemic cells which express the antigen to which M195 binds so as to thereby destroy the leukemic cells.

13. The method of claim 12, wherein the amount of therapeutic agent is from about 0.05 mg. to about 100 mg.

14. The method of claim 12, wherein the therapeutic agent is administered intravenously.

15. The method of claim 12, wherein the cytotoxic agent is Iodine-131 and Iodine-121 comprises an amount from about 50 mCi to about 200 mCi.

16. The method of claim 12, wherein the cytotoxic agent is Yttrium-90 and Yttrium-90 comprises an amount from about 10 mCi to about 50 mCi.

17. The method of claim 12, wherein the cytotoxic agent is Bismuth-212 and Bismuth-212 comprises an amount from about 20 mCi to about 80 mCi.

18. The method of claim 12, wherein the cytotoxic agent is Iodine-123 and Iodine-123 comprises an amount from about 100 mCi to about 300 mCi.

19. A method of destroying a human myeloid leukemia patient's bone marrow cells which comprises administering to the patient a therapeutic agent comprising humanized monoclonal antibody M195 (ATCC HB 10306) and a cytotoxic agent conjugated thereto under conditions such that the therapeutic agent binds to, and is internalized by, bone marrow cells which express the M195 antigen in an amount sufficient to destroy the patient's bone marrow cells.

20. The method of claim 19, wherein the amount of antibody is from about 0.01 mg to about 50 mg.

21. The method of claim 19, wherein the therapeutic agent is administered intravenously.

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The Preparation and Cytotoxic Properties of Antibody-Toxin Conjugates

PHILIP E. THORPE* & WALTER C. J. ROSS

I. INTRODUCTION

Interest has burgeoned recently in the possibility of attacking tumor cells with cytotoxic agents covalently linked to specific antibodies. This follows the innovation of cell hybridization techniques for producing monoclonal antibodies which has stimulated a world-wide search for antibodies with specificity for cancer cells. In anticipation that these endeavors will meet with success, a number of groups have attempted to devise ways of arming the antibody molecule to endow it with potent cytotoxic activity against the cells to which it can bind.

Our decision to use toxins as the cytotoxic component of the conjugate was provoked by their supreme potency. One molecule of the bacterial exotoxin, diphtheria toxin, or of the plant toxins, abrin, from *Abrus precatorius* or ricin, from *Ricinus communis*, may be sufficient to kill a cell which it penetrates (Yamaizumi et al. 1978, Eiklid et al. 1980). This maximizes the chance of killing cancer cells which do not express specific antigens at high density or which reside in solid tumors not freely permeable to the conjugate.

The modes of cytotoxic action of diphtheria toxin, abrin and ricin have been reviewed elsewhere (Collier 1976, Olsnes & Pihl 1976, Pappenheimer 1977, Gill 1978). Briefly, the toxins all comprise two polypeptide chains, denoted A and B, which are linked by a disulfide bond and which, when separated, are virtually devoid of cytotoxic action. The toxins bind via a recognition site on the B-chain to receptors on the cell surface and the A-chain then penetrates (or is translocated across) the cell membrane into the cytosol where it terminates protein synthesis. Diphtheria toxin A-chain inactivates elongation factor 2 by

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enzymically transferring to it the ADP-ribosyl moiety of NAD^+, whereas the A-chains of abrin and ricin damage the 60S subunit of the ribosome by a mechanism which has not yet been elucidated.

Two main approaches have been adopted for the synthesis of selective cytotoxic agents from antibodies and toxins (reviewed by Olsnes & Pihl 1981, Thorpe et al. 1982). The first is to link the intact toxin to the antibody molecule and to rely upon the specific binding properties of the bivalent antibody to override the non-specific binding properties of the toxin so that the net interaction is with the target cells. Part of the present review will concern the preparation and cytotoxic properties of such conjugates, particularly the way in which the different types of linkage used in constructing the conjugate influence its biological properties. We will also describe ways in which the non-specific cytotoxicity of conjugates with intact toxins can be reduced. A second approach is to link the isolated A-chain derived from a toxin directly to the antibody molecule. This manoeuvre generates conjugates capable only of binding to cells expressing the requisite antigens but, as will be seen, the cytotoxic potency of such conjugates is variable and at present unpredictable. A related approach is to make use of one of the single chain polypeptides which can be viewed as naturally-occurring toxin A-chains and which are to be found in the seeds of many plants (Gasperi-Campani et al. 1980). These inhibitors, which are virtually free from toxicity to intact cells, powerfully inactivate isolated eukaryotic ribosomes in a manner seemingly identical to that of the toxin A-chains. The cytotoxic and anti-tumor activities will be described for a conjugate containing one such inhibitor, gelonin, from *Gelonium multiflorum*.

II. PREPARATION OF ANTIBODY-TOXIN CONJUGATES

1. *Introduction*

The problem for the chemist is to form a covalent linkage between two dissimilar proteins to give a product of defined composition. In the present study a conjugate consisting of one molecule of antibody linked to one molecule of toxin was the objective since such simple conjugates were found usually to possess the maximal cytotoxic activity.

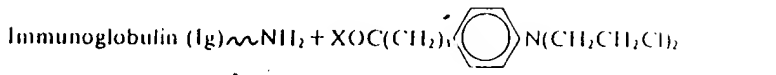
Until relatively recently the conjugation of dissimilar proteins was achieved by taking a solution containing the two components and adding a symmetrical difunctional agent such as a di-aldehyde, di-isocyanate, or di-imidoester. The resultant product was a mixture in which the homopolymer of one protein was often the main component. Intramolecular cross linking was also likely to occur between the A and B chains of the toxin (diphtheria toxin, abrin or ricin) thus hindering separation of the chains which is probably necessary for the toxic effect.

Various methods have been devised to overcome these problems and in our work we have concentrated on two approaches. In the first a heterobifunctional reagent is used in which the rate of reaction of the groups may be controlled by physical conditions (e.g. temperature, pH or irradiation) to permit the sequential assembly of the conjugate. This method is exemplified by the use of chlorambucil derivatives.

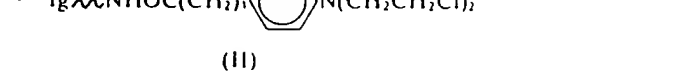
The second procedure involves attaching a group to the first protein which will react specifically with a group which is introduced into the second protein. Two examples of this approach are described. In each of these a thiol group is introduced into the antibody molecule and a group that reacts specifically with thiols is introduced into the toxin molecule.

2. *Conjugates prepared using chlorambucil derivatives*

The interest in this approach arose following the claims by Ghose et al. (1972) that non-covalent immunoglobulin-chlorambucil complexes showed enhanced anti-neoplastic activity. Since such complexes would be expected to dissociate *in vivo* (Ross 1974, Rubens 1974) it seemed desirable to prepare a covalent conjugate of chlorambucil (I, X = OH) with an immunoglobulin. This was originally achieved by adding a water-soluble carbodiimide to a cooled solution containing the globulin and chlorambucil (Ross 1974). Since this method is now known to produce globulin polymers the required conjugate (II) is best prepared by adding an activated chlorambucil derivative to a solution of the protein.



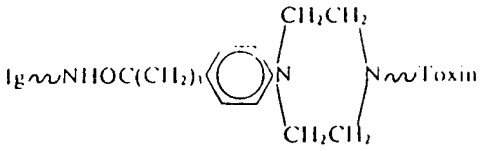
(I)



(II)

Initially a mixed anhydride (I, X = Bu^{n}O·CO·O) formed by reacting butyl to chloroformate with chlorambucil was employed but it was later found more convenient to use the N-hydroxysuccinimidylester of chlorambucil, which was isolated in pure form (Edwards et al. 1981b). The greater water solubility of this ester (I, X = $(CH_2CO)_2NO$) obviates the use of appreciable amounts of non-aqueous solvents in the reaction mixture and this has resulted in higher yields of more uniform products. The preparation of the conjugate (II) must be carried out at low temperature to avoid reaction of the chloroethyl-amino groups.

It occurred to us that a chlorambucil-antibody conjugate could be used as an intermediate in the covalent linkage of the antibody to a toxin molecule. When the purified conjugate (II) was mixed with toxin in borate buffer solution and the temperature raised, the di-2-chloroethylamino groups react predominantly with ε-amino groups in lysine side chains with the formation of a piperazine ring structure giving the antibody-toxin conjugate (III).



(III)

The reason for assuming that piperazine ring formation is the predominant reaction in buffer solution of pH 9 is that in model experiments using free chlorambucil and a globulin the number of primary amino groups masked (as estimated by the method of Habeeb 1966) corresponds closely to the number of chlorambucil moieties introduced. This is only consistent with piperazine formation and accords with the finding of Ross (1949) that in reaction with primary amines various NN-di-2-chloroethylarylamines afford only piperazines as identifiable products. The reaction involves other amino acids, such as aspartic and glutamic acids, if carried out at lower pH's.

Inevitably some reaction occurs between the chloroethylamino groups and the amino groups in the globulin molecule to generate antibody polymers, but this can be minimized by using an excess of toxin. An important feature is that the procedure carries little risk of cross-linking the A and B chains of the toxin.

We have investigated the effect of the chlorambucil loading of the immunoglobulin on the yield of toxin conjugate (III) and have found that in the

TABLE I

Introduction of chlorambucil groups into bovine γ-globulin using the ester (I, X = $(CH_2CO)_2NO$) Protein concentration 10 mg/ml in borate-saline buffer, pH 9, temperature 4°C, reaction time 1 h

Moles ester/mole protein used	Moles chlorambucil combined/ mole protein
3	2.5
6	5.1
12	8.0
24	13.2

range of 3 to 15 molecules of chlorambucil per molecule of globulin the yield of 1:1 conjugate (III) is reasonably constant, although with the higher loadings more polymerized high molecular weight material is produced.

Table I shows the degree of substitution when various amounts of chlorambucil ester (I, X = $(CH_2CO)_2NO$) are applied to bovine gamma globulin. It has been found convenient to use a loading of about 6–8 molecules of chlorambucil per globulin molecule in forming the primary conjugate (II). Little or no loss of the antigen-binding capacity of the antibody has been observed with this level of loading (Ross et al. 1980). The following is an example of a typical preparation

To a solution of horse anti-mouse lymphocyte globulin (3 ml, 10 mg/ml) borate-saline buffer (0.05M sodium borate, 0.3M NaCl, 0.5% Bu^{n}OH, pH 9.0) cooled to 4°C was added a solution of the N-hydroxysuccinimidyl ester of chlorambucil (1 mg) in dry dimethyl sulfoxide (0.5 ml). After stirring the cooled solution for 1 h, it was passed through a cooled column of Sephadex G-25 (1.6×36 cm), pre-equilibrated with the borate-saline buffer. The primary conjugate (II), which elutes soon after the void volume (25 ml), was run directly into a cooled solution of ricin (50 mg containing a small quantity of I^{125}-labeled toxin) in borate-saline buffer (5 ml). The final solution (20 ml) was concentrated to 5 ml in a cooled Amicon ultrafiltration cell using a PM 10 membrane and then allowed to reach room temperature. After maintenance for 2 days at this temperature the product was submitted to a chromatographic resolution (see section on purification procedures).

The yield of purified conjugate containing one molecule of toxin and one molecule of globulin was generally in the range of 5–10% based on the globulin used.

3. *Conjugates prepared using N-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia SPDP reagent)*

The use of this reagent (IV) is fully described by Carlsson *et al.* (1978). Essentially one introduces 2-pyridyl disulfide groups into both proteins giving the product (V). Treatment of one of the products with dithiothreitol (DTT) generates a thiol group giving (VI).

(IV) (V)

(VI)

On allowing a pyridyl disulfide substituted protein to react with a thiol substituted protein a conjugate (VII) is formed which has a central disulfide bond.

$$(V,\ P=P')+(VI,\ P=P'')-P'\sim NHOCCH_2CH_2SSCH_2CH_2CONH\sim P''$$
(VII)

In order to use this method we have studied the degree of substitution of globulins and toxins using the SPDP reagent (Table II). The use of borate-saline buffer solutions (pH 9) is preferable since it results in a higher degree of substitution than when phosphate-saline buffers (pH 7.5) are used and reaction with lysine ε-amino groups will predominate. The results obtained with a variety of N-hydroxysuccinimidyl esters and proteins (10 mg/ml in borate buffer) indicate that generally between 40 and 60% of the reagent reacts with the protein. The formation of polymeric material is restricted if the degree of substitution is kept low and we prefer to insert an average of one to two reactive groups for each molecule of protein. The thiol substituent is generated on the globulin rather than on the toxin molecule because there would be a risk of cleaving the toxin to its component A and B-chains if it was treated with the reducing agent. In all cases the final reaction of derivative (V) with derivative (VI) is conducted in phosphate-saline buffer (pH 7.5).

This method has also been used to prepare conjugates of abrin and ricin A-chains with immunoglobulins. Since the A-chains, prepared by reduction of the toxins, already contain a single thiol group they can be allowed to react directly with 2-pyridyl disulfide-substituted globulins. However, in preparing conjugates of gelonin, it is necessary to introduce a thiol group by the method indicated above.

TABLE II

Introduction of 2-pyridyldisulfide groups into proteins using the SPDP reagent. Protein concentration 10 mg/ml in borate-saline buffer, pH 9, temperature 20°C, reaction time 30 min

Protein	Moles SPDP/mole protein used	Moles 2-pyridyldisulfide combined/mole protein
Bovine γ-globulin	4.2	2.5
Normal horse IgG	3.6	2.3
	6.6	4.0
Horse anti-mouse IgG	3.6	2.0
Anti-Thy 1 antibody (IgG)	2.4	1.5
	6.0	5.0
Abrin	3.1	1.4
	5.2	3.0
Ricin	2.6	1.0
Gelonin	2.9	1.4

The following is an example of a typical preparation using our modification of the SPDP method.

To a solution of horse anti-mouse lymphocyte globulin (2.5 ml, 10 mg IgG/ml) in borate-saline buffer (pH 9) at room temperature was added a solution of SPDP reagent (0.187 mg) in dimethylformamide (30 μl). After stirring for 30 min the solution was passed down a column of Sephadex G-25 (1.6×36 cm) pre-equilibrated with acetate-saline buffer (0.1M sodium acetate, 0.1M NaCl, 1mM EDTA, 0.02% NaN_3, pH 4.5). Elution with the same buffer solution removed the substituted protein in 11 ml. Analysis of a 1 ml aliquot by the method of Carlsson et al. (1978) indicated that an average of 2 molecules of 2-pyridyl disulfide groups were introduced into each molecule of globulin.

A solution of SPDP reagent (0.15 mg) in dimethylformamide (24 μl) was added to a solution of abrin (1 ml, 10 mg/ml protein) in borate-saline buffer. After stirring for 30 min the solution was passed down a column of Sephadex G-25 (1.6×36 cm) pre-equilibrated with phosphate-saline buffer (0.1M sodium phosphate, 0.1M NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.5). Elution with the same buffer removed the substituted toxin in 11 ml. Analysis of the solution indicated that 1.4 molecules of 2-pyridyl disulphide were introduced into each molecule of toxin.

Ten ml of the solution of derivatized globulin was concentrated to 2.5 ml using an Amicon ultra-filtration cell and 22 mg of dithiothreitol was added. After stirring for 30 min at room temperature the solution was passed down a column of Sephadex G-25 pre-equilibrated with nitrogen flushed phosphate-saline buffer (see above). Elution with the same buffer removed the thiolated globulin in 11 ml and this was run directly into the derivatized abrin solution (10 ml). After concentrating to 5 ml the solution was left overnight and then submitted to a chromatographic resolution (see section on purification procedures).

The yield of purified conjugate containing one molecule of toxin and one molecule of globulin was between 10 and 15% based on the globulin used.

4. *Conjugates prepared using haloacetyl derivatives*

In this method one protein is thiolated and a reactive haloacetyl group is introduced into the second protein. On allowing the derivatized proteins to react a conjugate is formed which contains a central sulfide bond:

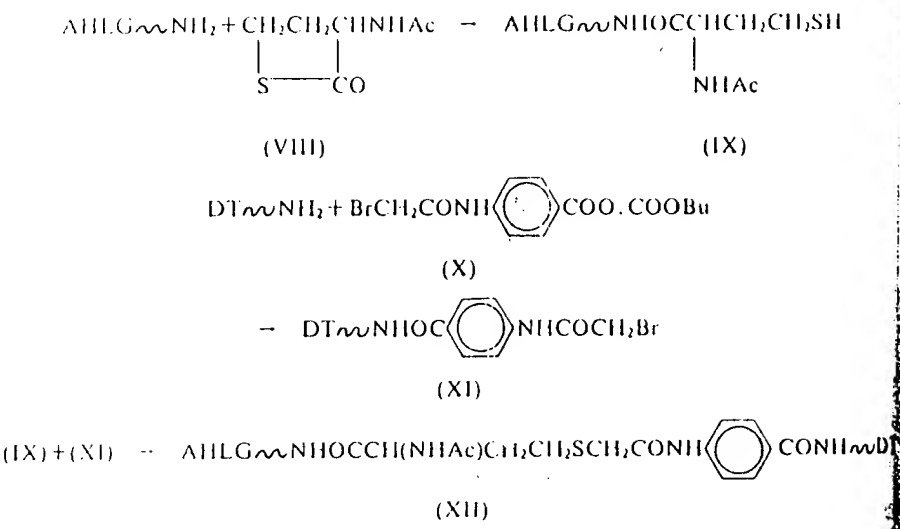
$$P'\sim SH+P''\sim COCH_2X\rightarrow P'\sim SCH_2CO\sim P''$$
(where X = halogen)

Two methods have been used for the thiolation stage. The first employed acetylhomocysteine thiolactone (VIII) giving the derivative (IX) and the second

used the SPDP procedure as described above. The second method is now preferred as it gives more reproducible results.

For the introduction of the reactive haloacetyl groups two methods have also been used. Firstly, activated bromoacetyl-p-aminobenzoic acid was employed – this has the advantage that an extra chromophoric group is introduced so that the degree of substitution can be determined by UV spectrophotometric methods. Originally we used a mixed anhydride (X) formed by the reaction of the acid with butyl chloroformate but, as with chlorambucil, we now prefer to use the N-hydroxysuccinimidyl ester of the acid.

The conjugate (XII) of horse anti-human lymphocyte globulin (AHLG) with diphtheria toxin (DT), for which biological results are given later, was prepared by our original method and so this is described below. The reactions involved are:

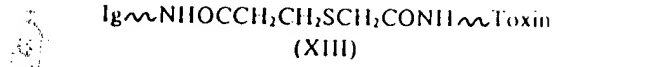


The experimental details for this procedure are as follows: To an ice-cooled solution of bromoacetyl-p-aminobenzoic acid (0.5 mg) and triethylamine (0.196 mg) in tetrahydrofuran (50 μl) was added butyl chloroformate (0.264 mg) in tetrahydrofuran (10 μl). After stirring for 30 min at 0°C a solution of diphtheria toxin (2 ml, 10 mg/ml) in borate-saline buffer (pH 9) was rapidly added and stirring continued for 1½ h. The solution was then passed through a column of Sephadex G-25 and the derivatized protein was eluted, as before, with borate saline buffer. Spectrophotometric analysis of the protein-containing eluate (11 ml) indicated that 2.75 molecules of bromoacetyl-amino acid had been introduced into each molecule of toxin.

Acetylhomocysteine thiolactone (0.25 mg) in water (50 μl) was added to an ice-cooled solution of horse anti-human lymphocyte globulin (2 ml, 10 mg/ml) in borate-saline buffer which had been adjusted to pH 10.7. After 20 min the solution was passed through a column of Sephadex G-25 pre-equilibrated with nitrogen-flushed borate-saline buffer. Elution with the same buffer removed the thiolated globulin in 11 ml and this was run directly into the solution of bromoacetylated toxin (see above). A preliminary run had indicated that this thiolation procedure introduced 1.25 thiol groups into each molecule of globulin, as analyzed by the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) method. After concentrating to 5 ml the reaction mixture was kept at room temperature for 24 h and then submitted to a chromatographic resolution.

The yield of the conjugate comprising one molecule each of antibody and toxin was 13–15% based on the globulin used.

More recently the introduction of an iodoacetyl group into ricin has been achieved using the N-hydroxysuccinimidyl ester of iodoacetic acid (cf. Rector et al. 1978) and the derivatized ricin has been coupled to immunoglobulins thiolated by the SPDP procedure. In this case the conjugates have the structure (XIII).



(XIII)

5. *Purification procedures*

The various reaction mixtures obtained by the methods described above were submitted to chromatographic resolution by gel filtration on columns previously calibrated with proteins of known molecular weight. At first we used Sephadex G-200 as the gel filtration media but as columns of this material are slow running we now prefer to use the faster running Sephacryl 200 and 300 media. With columns of these materials we routinely re-run the product of molecular weight about 215,000 daltons (corresponding to a 1+1 antibody-toxin conjugate) and this procedure effectively removes free toxin (Fig. 1).

It has also been found to be advantageous in the case of abrin and ricin conjugates further to purify the final product by affinity chromatography on Sepharose 4B, pre-treated with propionic acid. Any unconjugated globulin and globulin polymers are not retained by the column, whereas the abrin and ricin conjugates bind to the galactose residues of the Sepharose and can be eluted subsequently with solutions containing galactose.

It will be noticed that the final reaction mixtures are concentrated by ultrafiltration to a volume of about 5 ml. This has two advantages. Firstly, yields of conjugates are considerably reduced if this is not done and, secondly, this volume is suitable for direct application to our gel filtration columns. Butanol is

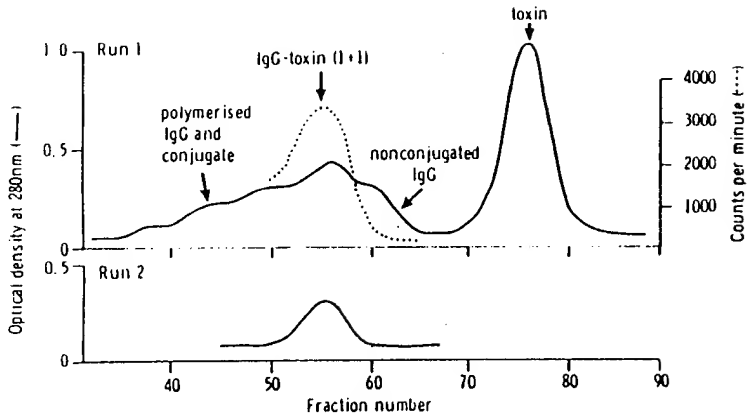


Figure 1. Purification of antibody-toxin conjugates on Sephacryl 300. The products of the reaction between ricin and anti-mouse lymphocyte globulin conjugated to chlorambucil were chromatographed on Sephacryl 300 (Run 1). Fractions 52–59, which contained the material with a molecular weight corresponding to a conjugate of one molecule each of antibody and ricin, were further purified by re-running on the same column (Run 2). The column eluates were monitored spectrophotometrically at 280 nm (—) and by radioactivity (·····) derived from the ^{125}I-labeled toxin.

included in the borate-saline buffers as an antimicrobial agent to keep the columns free flowing over prolonged periods at room temperature. The butanol must be removed (e.g. by dialysis) from solutions of conjugates if they are to be stored frozen as it prevents complete freezing and the proteins slowly denature. Eluates from the columns are monitored spectrophotometrically at 280 nm and by radioactivity, for in all instances the toxins are labeled with I^{125}.

6. *Calculation of the composition of the conjugates*

The following is an example of a typical calculation of the composition of a conjugate. The example has been taken from fraction 55, Run 2, Fig. 1.

Molecular weight of IgG = 150,000 daltons
Molecular weight of ricin = 65,000 daltons
$E^{1\%}_{1cm}$ at 280 nm of ricin = 1.18
$E^{1\%}_{1cm}$ at 280 nm of IgG = 1.40
O.D. at 280 nm of fraction 55 = 0.324
cpm on 1 ml sample of ricin used at 1 mg/ml = 26,500
cpm on 1 ml sample of fraction 55 = 2,010
Thus the concentration of ricin in fraction 55 = $2{,}010/_{26{,}500}$ or 0.0758 mg/ml

The O.D. due to the ricin content is $0.0758 \times 1.18 = 0.0895$
Therefore the O.D. due to the IgG content = 0.324–0.0895 or 0.2345 and the concentration of IgG = 0.2345/1.4 or 0.1675 mg/ml
The number of moles of ricin coupled to each mole of globulin is given by the expression:

$$\frac{\text{concentration of ricin} \times 150{,}000}{\text{concentration of IgG} \times 65{,}000}$$

i.e. $$\frac{0.0758 \times 150{,}000}{0.1675 \times 65{,}000}$$ or 1.04.

III. CYTOTOXIC PROPERTIES OF ANTIBODY-TOXIN CONJUGATES

1. *Conjugates with intact toxins*

i) *Diphtheria toxin:* This study was an extension of earlier work by Moolten and his colleagues who conjugated toxin to antibodies against viral antigens and against dinitrophenol and showed that the conjugates could protect hamsters from the growth of virally-transformed and haptenated tumor cells (Moolten & Cooperband 1970, Moolten et al. 1972, 1975).

Horse anti-human lymphocyte globulin (AHLG) was conjugated to diphtheria toxin using the mixed anhydride derivative of chlorambucil (Thorpe et al. 1978, Ross et al. 1980). Three fractions of conjugates were obtained with approximate molecular weights 210,000, 300,000 and 400,000, as judged from their elution volumes from a Sephadex G-200 column. The antibody component of the conjugate exhibited little or no diminution in its capacity to bind to antigens upon cells from the human lymphoblastoid lines, CLA4 and Daudi, as measured by an indirect immunofluorescence method (Ross et al. 1980).

The AHLG-toxin conjugates were highly toxic to Daudi cells in tissue culture, as shown by the marked impairment of the capacity of the cells to incorporate [^{3}H]-leucine into protein (Fig. 2). The most potent conjugate (M.210,000) was that comprising one molecule each of antibody and toxin. Treatment of the cells with this conjugate at a concentration of 8×10^{-12}M reduced their rate of leucine incorporation by 50%. The two other conjugates tested, with molecular weights of 300,000 and 400,000, were less active and toxin concentrations of 3×10^{-11}M and 4×10^{-11}M respectively were needed for the same effect upon protein synthesis. Hereafter, in our work with conjugates containing intact toxins, we standardized upon the use of conjugates comprising one molecule each of antibody and toxin.

In contrast to the potent cytotoxic action of AHLG coupled to diphtheria

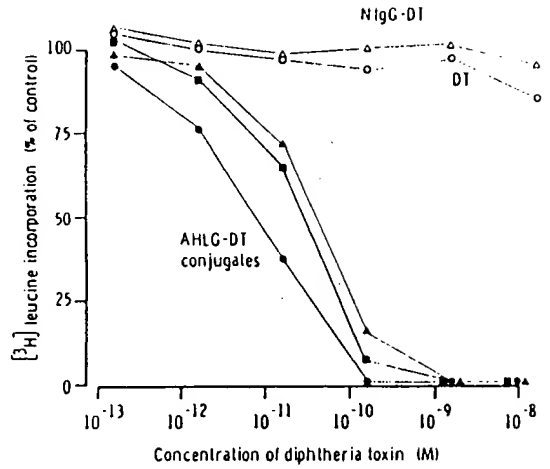


Figure 2. The cytotoxic effects of diphtheria toxin alone (○) and of conjugates with anti-human lymphocyte globulin or normal horse IgG (△) upon Daudi lymphoblastoid cells in tissue culture. The antibody-toxin conjugates tested had molecular weights corresponding to 210,000 (●), 300,000 (■) and 400,000 (▲). The control conjugate with normal IgG had an approximate molecular weight of 210,000. The cells were treated with the toxin or the conjugates for 24 h and their capacity to incorporate [^{3}H]leucine was assessed after a further 24 h period. The [^{3}H]leucine incorporation in the figure is expressed as a % of the incorporation in untreated cultures. Each point in the figure was calculated from the geometric mean of triplicate determinations, the standard deviations of which have been omitted for clarity but which were always less than 5% of the mean values in the figure. Reproduced by permission of the *European Journal of Biochemistry*.

toxin, neither diphtheria toxin alone nor a control conjugate with normal horse IgG exerted any cytotoxic action upon Daudi cells at the highest concentration of 1.6×10^{-8}M. Thus the toxicity of diphtheria toxin for Daudi cells had been improved more than one thousand times by its linkage to the specific antibody. Similar results have been obtained using AHLG-toxin against CLA4 cells (Thorpe et al. 1978) and against MOLT4 cells (Thorpe et al. 1982).

Control experiments showed that AHLG alone exerted no cytotoxic action and that when antibody and toxin were applied simultaneously to the lymphoblastoid cells, but not in chemically coupled form, no enhancement of toxicity occurred. Further, the specific cytotoxic effects of AHLG-toxin could be annulled by diphtheria antitoxin or by pretreating the cells with excess unconjugated AHLG. Lastly, the F(ab')$_2$ fragments of AHLG were as effective as a carrier for diphtheria toxin as the intact antibody. It was concluded from these experiments that the antibody moiety of the conjugates had facilitated the binding of a lethal quantity of diphtheria toxin to the target cells.

The magnitude of the increase in cytotoxic potency of diphtheria toxin for human lymphoblastoid cells following its linkage to the antibody owes much to the natural resistance of these cells to the toxin. Hitherto, all cells derived from toxin-sensitive species, apart from specially selected mutants, had been found to be highly sensitive to the toxin. The resistance of the lymphoblastoid cells could arise for two reasons. Firstly, it may be that diphtheria toxin, if it binds to cell surface receptors, is not delivered to a site in the cell where the A-chain can penetrate the cell membrane to gain access to the protein synthesis machinery. There is evidence that A-chain penetration occurs in an acidic cellular compartment, possibly the lysosomes, since chloroquine and other amines which raise lysosomal pH inhibit the toxic action of diphtheria toxin (Kim & Groman 1965) and cells exposed to the toxin at low pH are killed rapidly by a mechanism apparently not requiring endocytosis (Sandvig & Olsnes 1980, Draper & Simon 1980). Thus the AHLG-toxin conjugate may be toxic, whilst the free toxin is not, because the antibody stimulates the delivery of the toxin to the lysosomes by a mechanism possibly involving capping and endocytosis. A second explanation for the toxin resistance of the lymphoblastoid cells is that they are deficient in cell surface binding sites for the toxin; thus the attachment of AHLG restores the toxin's ability to bind and kill. This has the important implication that it may be possible to prepare wholly specific and highly cytotoxic conjugates from diphtheria toxin which has been chemically modified to destroy its cell binding properties. Alternatively, toxin mutants, such as CRM 45 (Uchida et al. 1973), which lack the portion of the B-chain concerned with cell binding, could be utilized.

An enigmatic finding was that spleen cells from mice, a species highly resistant to diphtheria toxin, were not killed by diphtheria toxin coupled to anti-mouse lymphocyte globulin (Ross et al. 1980). Since the conjugate was fully capable of binding to the cells and since it is known that elongation factor 2 isolated from mouse cells is inactivated by the toxin A-chain, this suggests that mouse cells lack a mechanism for translocating the A-chain across cell membranes into the cytoplasm.

The nature of the bond used to link together molecules of antibodies and diphtheria toxin greatly influences the cytotoxic activity of the conjugate. Two conjugates of AHLG and diphtheria toxin were prepared, one using a mixed anhydride derivative of chlorambucil (Section II-2) and the other using a bromoacetyl coupling agent (Section II-4). Neither conjugate displayed any loss of capacity to bind to Daudi cells. The conjugate formed with the chlorambucil derivative, abbreviated to AHLG-CB-DT in Table III, was 125 times as potent as the conjugate formed with the bromoacetyl derivative, abbreviated to AHLG-S-DT, at inhibiting protein synthesis by Daudi cells in tissue culture. When tested against the human fibroblast cell line, HS1, which lacks the antigens to which AHLG can bind, the chlorambucil-linked conjugate was

again seen to exert the greater cytotoxic effect. Both conjugates were more effective against the lymphoblastoid cells than against the fibroblasts (Thorpe et al., unpublished results).

The explanation for the inferior toxic action of the conjugate prepared with the haloacetyl derivative is obscure. The introduction of an average of 2.75 bromoacetylamino groups into diphtheria toxin, which is an intermediate step in forming the conjugate, had no deleterious effect upon its toxicity. This shows that acylation of amino groups in the toxin does not itself damage the enzymic activity of the A-chain, nor its cell binding and penetration properties, and that the loss of cytotoxic activity is a consequence of completing the linkage of the modified toxin to the antibody. Both coupling reagents react predominantly with lysine residues in the toxin and the antibody and the length of the linkages generated is comparable: thus there is no reason to expect the spatial arrangements of the conjugates to be greatly different. The possibility that more than one linkage can form between the same antibody and toxin molecules applies to both conjugation methods. There is no evidence that, after the conjugate binds to the cell surface, the intact toxin needs to be enzymically cleaved from the immunoglobulin in order to kill the cell. If, however, this is the case, then it must be postulated that the linkage generated by the chlorambucil derivative, which has only one amide bond as a potential site for hydrolytic cleavage, is more biologically labile than the linkage formed with haloacetyl derivative which has three amide bonds. This would not be too improbable in view of the specificity which some hydrolytic enzymes display in their choice of substrate (see Barrett 1977, Barrett & Heath 1977).

ii) *Abrin*: The F(ab')$_2$ fragment of monoclonal anti-Thy$_{1.1}$ antibody was coupled to abrin using a mixed anhydride derivative of chlorambucil. The conjugate was highly toxic to the Thy$_{1.1}$-expressing cells of the AKR lymphoma line, AKR-A,

TABLE III

Comparison of the cytotoxic effects of anti-human lymphocyte globulin (AHLG) conjugated to diphtheria toxin (DT) using a chlorambucil derivative (I) or a haloacetyl derivative (X)

	ID_{50}(M)		Selectivity
	Daudi	fibroblasts HS1	factor
DT	$>1.5\times10^{-9}$	1.5×10^{-11}	< 0.001
Bromoacetyl DT (XI)	$>1.5\times10^{-8}$	1.5×10^{-11}	< 0.001
AHLG-S-DT (XII)	6.4×10^{-10}	$>1.5\times10^{-8}$	>23
AHLG-CB-DT (III)	8.0×10^{-12}	1.5×10^{-9}	190

[a] ID_{50} = concentration of toxin or conjugate needed to inhibit the rate of protein synthesis by 50% after a 1 h period of exposure to the cells.

[b] Selectivity factor = ratio ID_{50} for fibroblasts: ID_{50} for Daudi cells.

[c] Roman numerals in parentheses refer to the full structures given in the text.

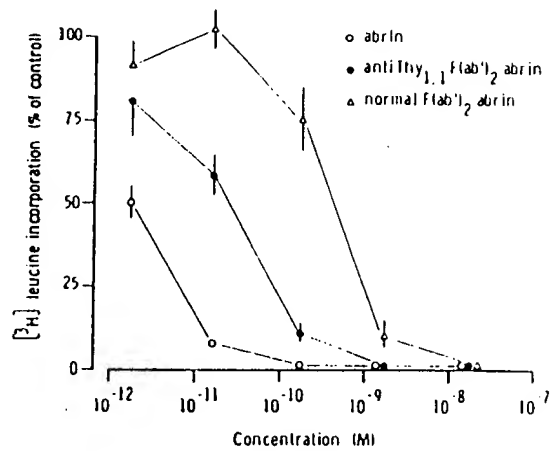


Figure 3. The cytotoxic effects of abrin alone (0) and of conjugates with the F(ab')$_2$ fragments of monoclonal anti-Thy$_{1.1}$ antibody (•) or of normal mouse IgG$_{2a}$ (Δ) upon AKR-A lymphoma cells in tissue culture. The cells were treated for 1 h with abrin or the conjugates and then were washed; and 23 h later 1 μCi of [^{3}H]leucine was added to the cultures. The [^{3}H]leucine incorporated during a 24 h period is expressed as a % of that in untreated cultures. Each point in the figure was calculated from the geometric mean of triplicate determinations, the standard deviations of which are represented by vertical lines unless smaller than the points as plotted. The conjugate corresponded by analysis to one molecule of antibody linked to one molecule of abrin. The hybrid cell line (clone T32B11) which secreted the monoclonal antibody was kindly provided by Drs. P. I. Lake and E. A. Clark, University College, London.

in tissue culture. Treatment of the cells for 1 h with anti-Thy$_{1.1}$F(ab')$_2$-abrin at a concentration of 2.5×10^{-11}M reduced the capacity of the cells to incorporate [^{3}H]-leucine into protein by 50% (Fig. 3). A control conjugate with abrin linked to the F(ab')$_2$ fragment of normal murine IgG$_{2a}$, the same subclass as the monoclonal antibody, was about 20 times less cytotoxic, showing that the greater potency of anti-Thy$_{1.1}$ F(ab')$_2$-abrin was mediated through its ability to interact with antigens upon the lymphoma cell surface. Both conjugates were less toxic than native abrin which reduced by half the capacity of the lymphoma cells to synthesize protein at a concentration of 1.8×10^{-12}M. Treatment of the cells with the antibody alone was ineffective (Thorpe et al., unpublished results).

The ability of anti-Thy$_{1.1}$F(ab')$_2$-abrin to protect immunologically-deprived mice against the growth of an AKR-A lymphoma allograft was investigated. The Thy$_{1.1}$ antigen expressed by the lymphoma cells is not found within CBA mice and so constitutes a tumor-specific marker in this model system. 10^4 lymphoma cells injected intraperitoneally into the mice will grow progressively, initially as an ascitic tumor, and kill the animals between 16 and 20 days later. Treatment of the mice with 1.5 pmoles of the conjugates (approximately half the LD_{50})

administered intraperitoneally 1 day after the tumor cells extended the median survival time by 5 days (Fig. 4). This therapeutic effect was insignificantly greater than that engendered by the control conjugate, normal $F(ab')_2$-abrin, which extended the median survival time by 4 days. Antibody alone afforded no protective effect. The therapeutic effect of anti-$Thy_{1.1}F(ab')_2$-abrin corresponds, as judged by calibration experiments, to an eradication of 99% of the tumor cells. When the number of lymphoma cells was reduced to 10^3 (equivalent to 100 lethal doses since 10 cells are needed to kill an animal), 40% of the mice were cured by their treatment with the conjugate. The conjugates, when administered intravenously rather than intraperitoneally, did not protect mice against the growth of lymphoma cells either in the peritoneum or implanted in the subcutaneous site. Thus the anti-tumor activity of the conjugate was disappointingly slight, and was only apparent when attacking very small numbers of tumor cells in the peritoneum with the conjugate delivered directly to this same site.

The immunosuppressive effect of treating mice with abrin conjugated to horse anti-mouse lymphocyte globulin (AMLG) has also been investigated. In their first two studies, Edwards et al. (1981a, b) used conjugates prepared using derivatives of chlorambucil. The conjugates were two to four times as effective

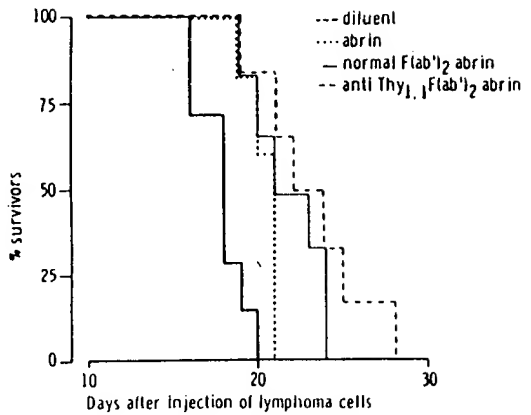


Figure 4. Prolongation of survival of immunologically-deprived CBA mice bearing a $Thy_{1.1}$-expressing lymphoma following the administration of anti-$Thy_{1.1}F(ab')_2$-abrin. The mice were injected intraperitoneally with 10^4 AKR-A lymphoma cells and 1 day later received an intraperitoneal injection of 1.5 pmoles of anti-$Thy_{1.1}F(ab')_2$-abrin (- - -) or normal mouse $F(ab')_2$-abrin (—) or 0.15 pmoles of abrin (····). Treatment of the mice with 1.5 pmoles of unconjugated anti-$Thy_{1.1}F(ab')_2$ produced no extension in their survival. The conjugates corresponded by analysis to one molecule of $F(ab')_2$ linked to one molecule of abrin. The hybrid cell line (T32BII) secreting the monoclonal antibody was kindly provided by Drs. P. L. Lake and E. A. Clark, University College, London.

as similar conjugates made with normal immunoglobulin (NIgG) at suppressing the ability of mice to produce specific IgM-antibody forming cells following an immunological challenge with sheep red blood cells. The conjugates more readily suppressed the generation of specific antibody-forming cells which secrete IgG than those which secrete IgM. Antibody alone was only immunosuppressive when injected in quantities 50,000 times greater than in the conjugate. The small superiority in the immunosuppressive potency of AMLG-abrin over that of NIgG-abrin appears, therefore, to be due to its ability to bind preferentially and kill cells which can engage in immune responses.

The type of linkage used to construct the AMLG-abrin conjugate influences its activity in tissue culture and its immunosuppressive activity in mice. Edwards et al. (1981c) tested two conjugates, one prepared using the N-hydroxysuccinimidyl ester of chlorambucil (AMLG-CB-abrin) and the other with the SPDP

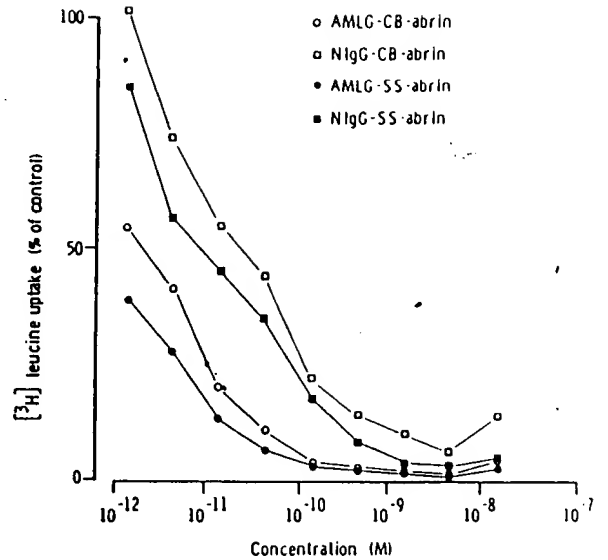


Figure 5. Comparison of the cytotoxic effects upon CBA T-lymphocytes of conjugates of anti-mouse lymphocyte globulin (AMLG) and abrin prepared by the use of either the N-hydroxysuccinimidyl ester of chlorambucil or the SPDP reagent. The conjugates, designated AMLG-CB-abrin (o) and AMLG-SS-abrin (•) respectively were compared with corresponding control conjugates with normal horse IgG (NIgG), designated NIgG-CB-abrin (□) and NIgG-SS-abrin (■). Spleen cells were treated with the conjugates for 1 h and were then washed and 23 h later concanavalin A was added to the cultures. The [^{3}H]leucine incorporation of the T-cells stimulated with the mitogen was measured after a further 24 h period and is expressed as a % of the incorporation in cultures not treated with the conjugates. Antibody alone at 10^{-8}M was without cytotoxic effect.

reagent (AMLG-SS-abrin) which introduces a disulfide bond between the two proteins. The A-chain of abrin in both conjugates was found to be fully capable of inhibiting protein synthesis in rabbit reticulocyte lysates showing that damage to the A-chains had not occurred during the coupling reactions. The conjugates were then compared with conjugates of normal horse IgG and abrin prepared by the same two methods for their capacity to inhibit protein synthesis by murine spleen cells in tissue culture, using the mitogen, concanavalin A, to stimulate the T-lymphocyte subpopulation (Fig. 5). Both AMLG-CB-abrin and AMLG-SS-abrin were 10 times as toxic as their appropriate control conjugates. However, both disulfide-linked conjugates were twice as toxic as their chlorambucil-linked counterparts. One explanation for the greater cytotoxicity of the disulfide-linked materials is that the abrin molecule could be attached to the immunoglobulin through either its A or its B-chain and both forms potentially could be cleaved by reduction inside the cell to release the active A-fragment; with the chlorambucil-linked conjugate, only the form with abrin linked by its B-chain to the globulin could release the A-chain by reduction. It is thought that A-chains linked by non-reducible bonds to antibody molecules are inactive (Jansen et al., personal communication).

In spite of its superior cytotoxicity in tissue-culture, the AMLG-SS-abrin conjugate was much inferior to AMLG-CB-abrin at immunosuppressing mice. An injection of 1.5 pmoles of the disulfide-linked conjugate had no discernable

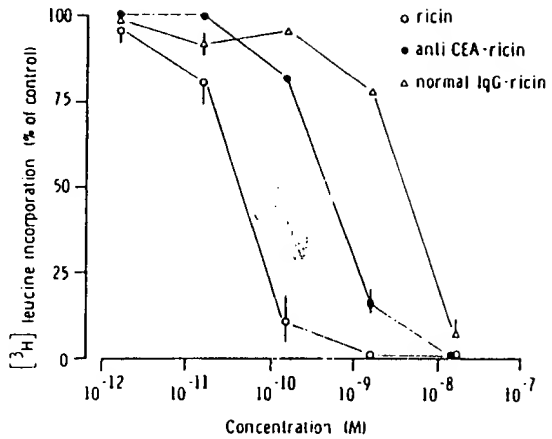
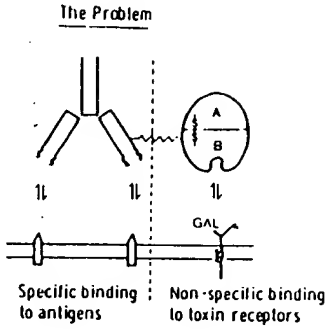


Figure 6. The cytotoxic effects of ricin alone (0) and of conjugates with immunopurified goat anti-CEA antibody (●) or normal goat IgG (Δ) upon the human colorectal carcinoma cell line, LOVO. Cultures of the cells were washed and were treated with the agents for 1 h before washing again. The [^{3}H]leucine incorporation by the cells was determined 24 h later. Other details are as in the legend to Fig. 3. The antibody was kindly provided by Dr. M. Ormerod, Ludwig Institute, Sutton, U.K.

Abrogation of the non-specific toxicity of antibody-abrin and antibody-ricin conjugates



Possible Solutions

1. Competitive antagonism of non specific binding with excess galactose or lactose
2. Chemical modification of the toxin to destroy its galactose-recognising properties by
   i. chemical alteration of selected amino acids
   ii. affinity labels
3. Conjugates with isolated A chains for gelonin)

Figure 7.

effect upon the number of IgM plaque forming cells produced per 10^6 spleen cells in mice immunized with sheep erythrocytes, whereas 0.38 pmoles of the chlorambucil-linked conjugates suppressed the capacity of the mice to respond to the immunogen by more than half. A similar difference was also observed in the capacity of the conjugates to reduce the levels of hemagglutinating antibody in the serum of the mice. Further, the disulfide conjugates were twice as poisonous to mice in acute toxicity tests. It was concluded that the disulfide bond, when used to couple two unrelated proteins, is not reinforced by non-covalent forces and may therefore be particularly susceptible to cleavage in animals either by reduction or by disulfide interchange with substances which contain free sulfydryl groups.

iii) *Ricin:* Carcinoembyronic antigen (CEA) is frequently, though not uniquely expressed by certain types of human cancer cells, particularly those of carcinomas originating in the colon (Gold & Freedman 1965, Fritsche & Mach 1977). An investigation was made into the possibility of preparing anti-cancer agents for the treatment of CEA-expressing malignancies by linking ricin to immunopurified goat antibodies against CEA (Thorpe et al., unpublished results). The conjugate, prepared using the N-hydroxysuccinimidyl ester of chlorambucil, was moderately cytotoxic to the CEA-expressing human colonic carcinoma line, LOVO, in tissue culture, reducing its rate of [^{3}H]-leucine incorporation by half at a concentration of 5×10^{-10}M (Fig. 6). A control conjugate comprising normal goat IgG and ricin was 10 times less toxic to the cells, indicating that interaction of anti-CEA-ricin with antigens upon the surface of the LOVO cells had facilitated the expression of its toxicity. Similar results were obtained with two other CEA-expressing cell lines, LS174 and Bentley.

The anti-CEA-ricin conjugate, when injected intravenously into immunologically-deprived mice bearing established subcutaneous xenografts of LOVO or LS 174 cells, did not delay the growth rate of the tumors even when used at doses approaching levels lethal to the animals.

2. *Abrogation of the non-specific toxicity of antibody-toxin conjugates*

Conjugates of antibodies and intact toxins can bind to cell surfaces by two mechanisms. In addition to being able to interact via the antibody moiety with antigens upon the target cells, they can also interact nonspecifically via the recognition site on the B-chain of the toxin moiety with receptors which are upon the surface of most cell types (Fig. 7). The tendency of the conjugate to bind via the specific as opposed to the nonspecific route is determined both by the extent to which the affinity of the antigen-antibody interaction exceeds that of the toxin-receptor interaction and by the relative abundance of the antigens and toxin receptors. In tissue culture, where many, if not all, of the cells express the antigens to which the antibody can bind, the predominant interaction tends to be via the specific binding route, as evidenced by the fact that antibody-toxin conjugates normally display greater toxicity for target cells than do conjugates with normal immunoglobulin. In animals, however, where the target cells usually are vastly outnumbered by cells lacking the appropriate antigens, the conjugates seem predominantly to bind non-specifically. Thus the conjugates are highly poisonous to animals and show little selectivity of cytotoxic action upon target cells within them.

The problem is to eliminate the binding of the toxin moiety of the conjugate to the receptor molecule but to do so in such a way that conjugates which attach to specific antigens can still express their toxic effects. Three possible solutions to the problem of the non-specific toxicity of conjugates with abrin or ricin are listed in Fig. 7 and will be detailed below.

i) *Competitive antagonism of the non-specific toxicity of antibody-abrin and antibody-ricin conjugate with galactose or lactose:* One way of abrogating the non-specific toxicity of antibody-toxin conjugates to cells in tissue culture is competitively to antagonize the binding of the toxin moiety to its receptor molecule. No such inhibitors are known for diphtheria toxin, but the binding of abrin and ricin, which are known to recognize galactose-containing glycoproteins and glycolipids upon cell surfaces, can be antagonized with excess free galactose or lactose. The affinity of interaction of these simple sugars with the toxins (K_a of around $10^4 M^{-1}$) is much lower than that with the cell surface receptors, (K_a of $3 \times 10^7 M^{-1}$ at 0°C for HeLa cells) so that for good protection from toxicity high concentrations of sugars are needed (Olsnes et al. 1974, Sandvig et al. 1976).

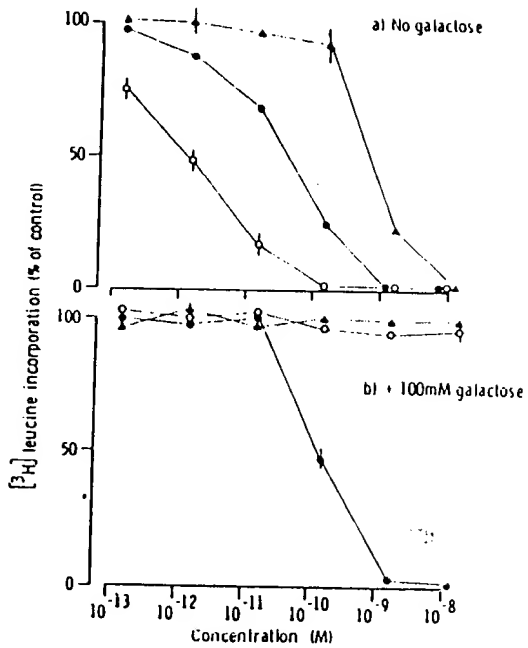


Figure 8. Abrogation by galactose of the non-specific cytotoxic effects of an antibody-abrin conjugate. Daudi lymphoblastoid cells were incubated for 1 h, in the presence (b) or absence (a) of 100mM galactose, with abrin alone (○) or with abrin conjugated to anti-human lymphocyte globulin (●) or to normal horse IgG (▲). The capacity of the cells to incorporate [^{3}H]leucine was measured 1 day later. Other details as for the legend to Fig. 3. Reproduced by permission of Blackwell Scientific Publications Ltd.

In the absence of galactose, anti-human lymphocyte globulin (AHLG) conjugated to abrin was only 10 times more toxic to Daudi cells in tissue culture than a control conjugate containing normal IgG, and both conjugates were less effective than free abrin. However, when 100mM galactose was included in the culture medium, the toxic effects of abrin and normal IgG conjugated to abrin were completely abolished, whereas that of the AHLG-abrin conjugate was unimpaired (Fig. 8) (Thorpe et al. 1981a). Similar results have been described for ricin conjugates by Youle & Neville (1980).

The degree of protection from the toxic effects of abrin or ricin which is afforded by 100mM galactose or lactose varies according to the cell type. The toxicity of abrin to the murine thymic lymphoma, TLX5, and the Daudi cell line is antagonized by more than 1000-fold. By contrast, murine and rat spleen cells are harder to protect, the toxicities of abrin and ricin being diminished only by

10 to 100-fold. This presumably reflects differences in the glycoproteins or glycolipids associated with the plasma membranes of the different cells and in the strength of their interaction with the toxins. Alternatively, abrin and ricin can also bind to certain types of cells by a mechanism not requiring the recognition of galactose.

ii) *Conjugates with abrin modified chemically to block its galactose-binding site.* An attractive idea is to modify a toxin chemically to destroy its cell surface recognition properties and then, by linking the modified molecule to an antibody, provide it with a new means of attaching to and killing cells with a specificity determined solely by the antibody.

The modification of abrin or ricin could be achieved, for instance, by the use of affinity labels prepared by the addition of a chemically reactive group to galactose or lactose. The affinity of interaction of these sugars with the toxins is rather weak and it may be advantageous to utilize complex glycoproteins, such as those from asialofetuin, which interact more strongly with the toxins (Baenziger & Fiete 1979).

Another approach is to use reagents which selectively modify certain amino acids with the aim of distorting the cell binding site of the toxin without damaging the activity of its A-chain or its cell membrane penetration qualities. In confirmation of an earlier study by Sandvig et al. (1978), we have found that the treatment of abrin with reagents which modify lysine or tyrosine residues reduces its ability to bind to galactose (as assessed by its reduced binding to sepharose) without influencing the activity of the A-chain as an inhibitor of protein synthesis. One such reagent, the SPDP reagent, which reacts primarily with lysine residues, can be used not only to block the galactose-binding properties of abrin, but also to couple the modified toxin to an antibody molecule.

The SPDP reagent was used to introduce 2-pyridyl disulfide groups into abrin at an average loading of 3.5 groups per molecule of the toxin. A proportion of the modified abrin molecules displayed a reduced ability to adsorb to the galactose residues of sepharose. When chromatographed on a sepharose column, three fractions of abrin-SSPy were obtained: one, fraction 1, passed unretarded through the column; a second, fraction 2, was retarded by the column but eluted without the application of galactose, and a third, fraction 3, bound to the column and was later displaced with 0.01M galactose. All three fractions fully retained their capacity to inhibit protein synthesis in rabbit reticulocyte lysates, showing that the modification had not damaged the A-chain activity.

The toxicity of fraction 3 to mice was comparable with that of native abrin, whereas fractions 1 and 2 were less toxic than abrin by 15 and 10-fold respectively. When tested for cytotoxicity to Daudi cells in tissue culture,

fractions 1 and 2 were 125 and 61 times less potent than native abrin but, this time, fraction 3 also showed some loss in activity, being seven times less potent than abrin (Table IV). The toxicity of fraction 1 to the Daudi cells was only weakly antagonized by the incorporation of 100mM lactose into the cultures, whereas the effects of native abrin and abrin-SSPy fraction 3, were strongly inhibited. Also, fraction 3 hemagglutinated human erythrocytes whilst fraction 1 did not. The reduction in the toxicity of fractions 1 seems, therefore, to derive from an impairment in its capacity to attach to galactose-containing molecules upon the cell surface.

As was seen with the Daudi cells, fraction 1 was found to be 100 times less effective than fraction 3 at inhibiting protein synthesis in cultures of AKR spleen cells to which concanavalin A was later added to stimulate the surviving T-lymphocytes (Fig. 9). Conjugates of modified abrin, fractions 1 and 3, were then prepared by their reaction with monoclonal anti-$Thy_{1.1}$ antibody to which a thiol group had been introduced.

The toxicity of fraction 1 for $Thy_{1.1}$-expressing AKR splenic T-lymphocytes was partially restored by its linkage to the antibody, whereas that of fraction 3 was diminished so that both conjugates inhibited the leucine incorporation by the cells by 50% at a concentration of 6×10^{-10}M (Fig. 9). The toxic action of the conjugates was not inhibited by the incorporation of 100mM lactose into the cultures (results not shown). One explanation for these results is that the modified abrin, fraction 1, with the blocked galactose-recognition site had been provided, by its linkage to the antibody, with a new means of attaching to and killing lymphocytes. Implicit in this suggestion is that the galactose-binding site of abrin serves no purpose other than to attach the toxin to cell surfaces and does not mediate the penetration of the A-chain through cell membranes, for instance by interacting with a galactose-containing transport molecule.

These results are in contrast with those of Youle et al. (1981), who found that ricin, when O-acetylated to reduce its sepharose and cell binding properties, was rendered 10 times less toxic to cultured human fibroblasts, but that a conjugate of acetylated ricin and the fibroblast-binding molecule, mannose-6-phosphate, was also 10 times less toxic than a conjugate with native ricin. It may be that the blockade of the galactose-binding site of the abrin conjugate described above is removed, possibly enzymically, after the conjugate has attached to the lymphocyte surface. Alternatively, Man6P-ricin but not anti-$Thy_{1.1}$-abrin needs to interact with a galactose-containing receptor molecule upon the cell surface or within an endocytic vesicle in order for the A-chain to penetrate the cell membrane.

An enigmatic finding was that the conjugates of anti-$Thy_{1.1}$ antibody and modified abrin, fractions 1 and 3, possessed similar abilities to exert non-specific cytotoxic effects upon spleen cells which lack the $Thy_{1.1}$ antigen, namely the B-lymphocytes from AKR mice which will respond to bacterial lipopolysaccharide

TABLE IV

The properties of abrin modified by treatment with the SPDP reagent

	Sepharose binding properties	Haemagglutination titer[a]	A-chain activity[b] ID_{50} (nmoles/ml)	Toxicity to mice[c] MLD_{100} (pmoles/25g mouse)	Toxicity to Daudi cells in culture[d] ID_{50} without lactose	ID_{50} with 100mM lactose	Toxicity inhibition factor with lactose
Abrin	bound	7	0.15	0.61	0.10	6.1	61
Abrin-SSPy (fraction 1)	not bound	>70	0.15	9.2	12.5	38	3
Abrin-SSPy (fraction 2)	retarded	N.D.	0.15	6.1	6.1	49	8
Abrin-SSPy (fraction 3)	bound	11	0.15	0.76	0.70	15	23

[a] Minimal concentration of abrin which haemagglutinates neuraminidase-treated human erythrocytes (blood group A) – performed by Dr. J. A. Forrester, Institute of Cancer Research.

[b] Concentration of abrin after reduction with dithiothreitol which inhibits by 50% the rate of protein synthesis in rabbit reticulocyte lysates – performed by Mrs. D. McIntosh, Institute of Cancer Research.

[c] Minimal dose of abrin injected intraperitoneally to kill 100% of mice.

[d] ID_{50} = concentration of abrin which inhibits the ^3H-leucine incorporation by Daudi cells by 50%. Toxicity inhibition factor = ratio ID_{50} (with lactose): ID_{50} (without lactose).

Figure 9. The cytotoxic effects upon AKR T-lymphocytes of abrin which had been chemically modified to impair its galactose-binding properties are partially restored by its linkage to anti-Thy1.1 antibody. Abrin, after its treatment with the SPDP reagent, was resolved into abrin-SSPy, fraction 1 (○), which passed through a sepharose column, and abrin-SSPy, fraction 3 (△) which was retained by the column. AKR spleen cells were treated for 1 h with monoclonal anti-Thy1.1 antibody conjugated to abrin-SSPy, fraction 1 (●) or to abrin-SSPy, fraction 3 (▲) or with the abrin-SSPy fractions from which the conjugates were derived. The conjugates and abrin derivatives were applied to the cells in Dulbecco's phosphate-buffered saline without serum but with 10 mg/ml human gamma globulin to deter non-specific binding of the conjugates. Twenty-four hours later the spleen cells were stimulated with the T-lymphocyte mitogen, concanavalin A, and the [^{3}H]leucine incorporation by the cultures was determined after a further 24 h period. The [^{3}H]leucine incorporation is expressed as a % of that in cultures not treated with conjugates or abrin derivatives. Antibody alone at 10^{-7}M was without inhibitory effect. The hybrid cell line (T32B11) which secreted the monoclonal antibody was kindly provided by Drs. P. I. Lake and E. A. Clark, University College, London.

and the T-lymphocytes from CBA mice. The cells were only about 10 times less sensitive to the conjugates than were the AKR-lymphocytes. Therefore, no improvement in the selectivity of action of anti-Thy1.1 F(ab')$_2$-abrin upon Thy1.1-bearing lymphocytes had apparently been achieved as a result of blocking the galactose-binding properties of the toxin component. It seems as though the conjugates have a means of attaching to the non-antigen-bearing lymphocytes by a route not requiring recognition of galactose, since the non-specific effects of the conjugates were not inhibited by the inclusion of 100mM galactose in the cultures (results not shown).

3. *Conjugates with toxin A-chains or gelonin*

In many laboratories, conjugates have been prepared by linking the A-chain isolated from ricin or diphtheria toxin directly to the antibody molecule (reviewed by Olsnes & Pihl 1981, Thorpe et al. 1982). Disulfide linkages have nearly always been employed to mimic the bond naturally occurring between the A and B-chains of the native toxin. Such conjugates, lacking the toxin B-chain, should only attach to cells which express the appropriate antigens upon their surfaces and so display great selectivity in their cytotoxic actions. The drawback is that the B-chain, besides its role in cell binding, may mediate the penetration of the A-chain through cell membranes, a function which may be replaced inefficiently or not at all by the antibody molecule.

i) *Diphtheria toxin A-chain:* Monoclonal anti-$Thy_{1.1}$ antibody was linked to the A-chain from diphtheria toxin using the SPDP reagent and the conjugate comprising one molecule each of antibody and A-chain was purified. When examined by an indirect immunofluorescence method (Ross et al. 1980), its capacity to bind to AKR thymocytes was found to be fully retained.

The conjugate inhibited protein synthesis by the $Thy_{1.1}$-expressing murine lymphoma cell line, BW5147, by 50% at a concentration of 10^{-7}M. Fragment A alone was less effective, a concentration of 4.5×10^{-6}M inhibiting the rate of protein synthesis of the cells by only 30%. Unconjugated antibody at 10^{-5}M was without toxic effect, nor did it act synergistically with free A-chain. Similar results were obtained when using the conjugate against AKR splenic T-lymphocytes and against AKR-A lymphoma cells.

Although the covalent linkage of diphtheria toxin fragment A and anti-$Thy_{1.1}$ antibody yielded a conjugate with a cytotoxic potency greater than that of the two parent proteins, it is important to note that its effectiveness was far less than that normally attributable to intact diphtheria toxin when used against toxin-sensitive cells.

These results accord with those obtained using diphtheria toxin A-chain linked to concanavalin A (Gilliland et al. 1978), *Wisteria floribunda* lectin (Uchida et al. 1978), anti-L1210 Fab' (Masuho et al. 1979), anti-concanavalin A (Gilliland & Collier 1980), anti-human colorectal carcinoma (Gilliland et al. 1980), anti-$H\text{-}2^k$ anti-$Thy_{1.2}$ (Martinez et al. 1981) and insulin (Miskimins & Shimizu 1979) which all exhibited limited cytotoxicity to cells to which the carrier could bind. Diphtheria toxin A-chain coupled to monophosphopentamannose (Youle et al. 1979) or to human placental lactogen (Chang et al. 1977) was, however, without cytotoxic effect upon human fibroblasts or lactating mammary gland respectively. Similarly, a conjugate of epidermal growth factor and this A-chain failed to inhibit protein synthesis by 3T3 cells to which it was demonstrated to bind (Cawley et al. 1980b). The only extremely potent conjugate containing diphtheria toxin A-chain which has been described so far been is that with asialofetuin and this inhibited protein synthesis by primary rat hepatocytes at concentrations as low as 10^{-11}M after binding to their receptors for galactose-terminated glycoproteins (Cawley et al. 1981) (see Discussion below).

ii) *Ricin A-chain:* The monoclonal antibody, W3/25, which recognizes an antigen expressed by the subpopulation of rat T-lymphocytes to which 'helper' cell function has been ascribed (Mason et al. 1980) was linked by a disulfide bond to ricin A-chain. The coupling was achieved using the SPDP reagent described earlier (Section II-3) and the component comprising one molecule each of antibody and A-chain was purified. The conjugate was found fully to retain its antigen-binding capacity, as demonstrated by its ability to compete equally with radiolabeled native W3/25 antibody for antigens upon rat T-lymphocytes. The ribosome-damaging properties of the A-chain in the conjugate were also completely preserved.

The conjugate was found not to inhibit the capacity of rat spleen cells to respond in tissue culture to phytohemagglutinin, a mitogen which predominantly stimulates the W3/25-expressing T-lymphocyte subpopulation (M. Webb, personal communication). Even at a concentration of 3×10^{-8}M, at which the antibody moiety should have saturated the antigens upon the cells to which it bound (Mason & Williams 1980), the conjugate caused no impairment in their rate of [^{3}H]-leucine incorporation (Fig. 10). Further, no inhibition in the phytohemagglutinin response was observed after the spleen cells had been coated with both W3/25-ricin A-chain and another conjugate containing the monoclonal antibody, MRC OX8, which binds to all those splenic T-lymphocytes which lack the W3/25 antigen (Mason et al. 1980). By contrast, ricin itself was highly toxic and reduced the [^{3}H]-leucine incorporation by the cells in response to phytohemagglutinin by 50% at a concentration of 10^{-11}M.

There is suggestive evidence that ricin needs to be endocytozed in order for its A-chain to penetrate a cell membrane and kill the cell (Olsnes et al. 1976, Sandvig & Olsnes 1979). Monoclonal IgG antibodies often recognize a single antigenic determinant upon the molecules to which they bind and, therefore, can cross-link only two cell surface molecules, which is insufficient to cause capping and endocytosis of the complex (Roser & Howard, unpublished observations). It was therefore not surprising to find that the W3/25-ricin A conjugate, when visualized by indirect immunofluorescence, was evenly distributed upon the rat T-lymphocyte plasma membrane with no indication of capping and endocytosis even after prolonged incubation at 37°C.

The possibility that the lack of toxicity of the W3/25-ricin A conjugate to the T-cells was due to its failure to be endocytozed was investigated by applying rabbit anti-mouse immunoglobulin antibodies to rat spleen cells which had been

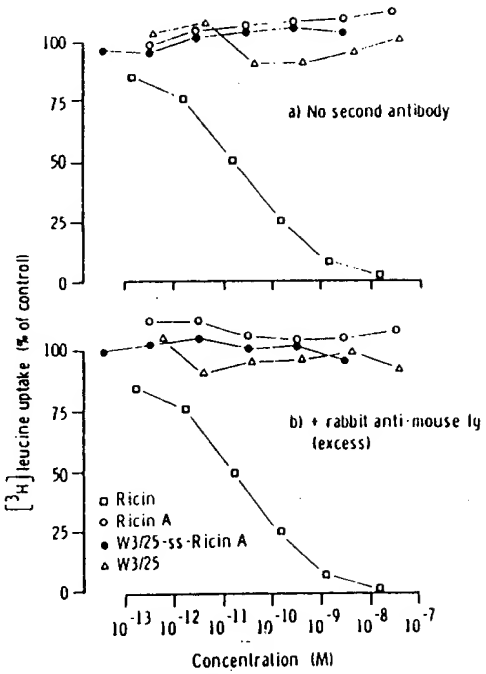


Figure 10. Ricin A-chain coupled to a monoclonal antibody, W3/25, is not toxic to rat T-lymphocytes in tissue culture even when endocytosis of the conjugate is stimulated with rabbit anti-mouse IgG. Hooded rat spleen cells were treated for 1 h at 37°C with ricin (□), ricin A-chain (○), W3/25 antibody (Δ) or the W3/25-ricin A-chain conjugate (●) and were then washed. The cultures were then incubated for 24 h in the presence (b) or absence (a) of 20 μg/ml of immunopurified rabbit anti-mouse IgG. Phytohemagglutinin was added to the cultures and, after a further 24 h, the [^{3}H]leucine incorporation of the cells stimulated with the mitogen was determined, and this is expressed as a % of the incorporation in cultures not treated with the conjugate or its components. The monoclonal antibody and the rabbit-anti-mouse IgG were kindly provided by Dr. D. W. Mason, MRC Cellular Immunology Unit, Oxford, U.K.

coated with the conjugate. The second antibody was demonstrated by indirect immunofluorescence to cause rapid aggregation and internalization of the conjugate. However, the rat spleen cells treated in this manner still showed no impairment in their capacity to respond to phytohemagglutinin in tissue culture (Fig. 10).

Ricin A-chain in disulfide linkage with antibodies and other carrier molecules has been found to vary markedly in cytotoxicity. Oeltmann & Heath (1979) failed to inhibit Leydig cells with a conjugate of ricin A-chain and the β-subunit of human chorionic gonadotrophin. Most workers, however, have reported

conjugates that are capable of expressing limited cytotoxicity to the cells to which they bind, but with a potency much less than that of native ricin. This was the finding with conjugates containing antibodies against immunoglobulin determinants (Raso & Griffin 1980, Miyazaki et al. 1980, Krolick et al. 1980), L1210 leukemia cells (Masuho & Hara 1980), human colorectal carcinoma cells (Gilliland et al. 1980) and H-2^k histocompatibility antigens (Martinez et al. 1981). In other instances, conjugates with ricin A-chain have mediated selective cytotoxic effects with a potency rivalling that of the native toxin, as was seen with Blythman's anti-Thy$_{1.2}$ monoclonal antibodies and Jansen's anti-DNP antibodies and with conjugates which used as the carrier *Wisteria floribunda* lectin (Uchida et al. 1980), concanavalin A (Yamaguchi et al. 1979), asialofetuin (Cawley et al. 1980a) and epidermal growth factor (Cawley et al. 1980b).

iii) *Gelonin:* In contrast to our disappointing results described above for the conjugates containing ricin or diphtheria toxin A-chains, gelonin linked by a disulfide bond to monoclonal anti-Thy$_{1.1}$ antibody was an extremely potent and specific cytotoxic agent (Thorpe et al. 1981b). This work was an extension of that by Stirpe et al. (1980), who showed that gelonin could acquire weak cytotoxic activity by its linkage to concanavalin A.

The SPDP reagent was used to couple together the antibody and gelonin and yielded two conjugate fractions with molecular weights 180,000 and >200,000. The conjugates, after cleavage with dithiothreitol, were found to have retained only 16% and 9% respectively of the ability of native gelonin to inhibit protein synthesis in rabbit reticulocyte lysates; the coupling procedure had, therefore, partially inactivated the gelonin.

The conjugates powerfully inhibited those Thy$_{1.1}$-bearing T-lymphocytes from AKR mice which will respond to phytohemagglutinin and concanavalin A in tissue culture. The [^{3}H]leucine incorporation in cultures stimulated with the T-cell mitogens was reduced by 50% following treatment with the 180,000 M, fraction at a concentration of 4×10^{-10}M or with the 200,000 M, fraction at 10^{-12}M (Fig. 11). Free gelonin induced comparable suppression of the T-cell responses at 3×10^{-8}M and antibody alone at 10^{-6}M was without effect. The conjugates at a concentration of 10^{-6}M exerted little or no toxic action upon lymphocytes which lack the Thy$_{1.1}$ antigen, including B-lymphocytes from AKR mice and T-lymphocytes from CBA mice. Thus it appears that gelonin, by its linkage to anti-Thy$_{1.1}$ antibody, acquired a cytotoxic potency which matched or exceeded that of abrin and ricin, but which was exercised only upon lymphocytes bearing the Thy$_{1.1}$ antigen.

This result is even more remarkable in view of the fact that about 90% of the gelonin in the conjugate appears to have been inactivated by the conjugation procedure so that the cytotoxic effects observed may have been due to only a minority of active conjugate molecules.

In further work, anti-$Thy_{1.1}$ antibody has been coupled to gelonin using the N-hydroxysuccinimidyl derivative of chlorambucil which generates a linkage insusceptible to cleavage by reduction. This method produced conjugates with a wide range of molecular weights, as judged by chromatography upon sephacryl S300. Fractions with approximate molecular weights of 180,000, 350,000, 600,000 and ≥1,000,000 were submitted to biological testing. The gelonin in the

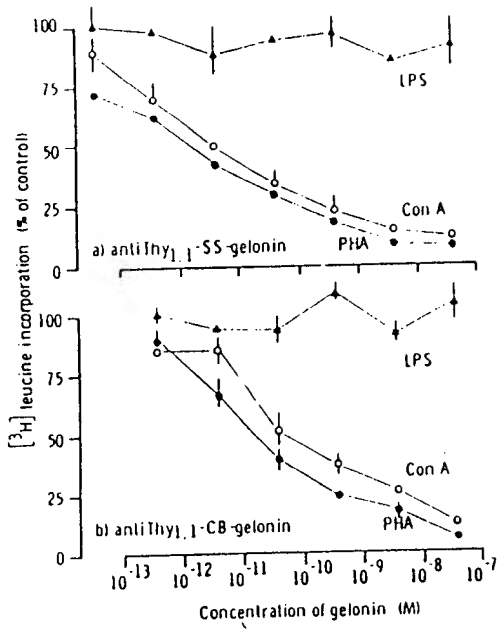


Figure 11. Comparison of the cytotoxic effects upon AKR T-lymphocytes of conjugates of monoclonal anti-$Thy_{1.1}$ antibody and gelonin prepared by the use of either the SPDP reagent (a) or the N-hydroxysuccinimidyl ester of chlorambucil (b). The conjugate made with the SPDP reagent, anti-$Thy_{1.1}$-SS-gelonin, had a molecular weight of 200,000 and the anti-$Thy_{1.1}$-CB-gelonin conjugate made by the chlorambucil method had a molecular weight of ≥1,000,000. Two other chlorambucil-linked conjugates with molecular weights 350,000 and 600,000 were only slightly less potent than the conjugate described in the figure (see text). AKR spleen cells were incubated for 1 h with the conjugates and 1 day later received either the B-cell mitogen, bacterial lipopolysaccharide (▲) or one of the T-cell mitogens concanavalin A (○) or phytohemagglutinin (●). The [^{3}H]leucine incorporation stimulated by the mitogens was measured after a further 24 h period and is expressed as a % of the incorporation in cultures not treated with the conjugates but stimulated with the corresponding mitogen. The antibody alone at 10^{-8}M was without inhibitory effect. The hybrid cell line (T32B11) secreting the monoclonal antibody was kindly provided by Drs. P. E. Lake and E. A. Clark, University College, London.

various conjugated fractions was found to have retained only 3 to 4% of the capacity of native gelonin to inhibit protein synthesis in reticulocyte lysates.

All four conjugate fractions strongly inhibited those AKR spleen cells which will respond to the T-cell mitogens, phytohemagglutinin and concanavalin A, and spared those which respond to bacterial lipopolysaccharide, a B-cell stimulant. The concentrations of the conjugates, in order of increasing molecular weight, which reduced by 50% the leucine incorporation of the T-lymphocytes were 2×10^{-9}M, 6×10^{-11}M, 3×10^{-11}M, and 2×10^{-11}M with respect to gelonin content. The conjugates were thus about 20 times less potent than those of comparable molecular weight which had been formed using the SPDP reagent (Fig. 11). It is possible that this factor is due to differences in the extent to which gelonin is inactivated by the two conjugation methods and not to the influence of the type of linkage upon the cytotoxic activity of the conjugates.

The marked inhibitory activity of the chlorambucil-linked conjugate contrasts with the results of Jansen et al. (personal communication) who found that monoclonal anti-DNP linked to ricin A-chain by a thioether bond, which is also insusceptible to cleavage by reduction, was devoid of cytotoxic activity. A comparable disulfide linked conjugate was highly effective. It may be that gelonin either does not need to be cleaved from anti-$Thy_{1.1}$ antibody in order to act, or that cleavage is mediated through enzymic hydrolysis, perhaps within the lysosomes. Alternatively, gelonin and ricin A-chains utilize different translocation mechanisms. The apparent requirement for a disulfide linkage in ricin A-chain conjugates could be, for instance, because there is a thiol-containing transport protein, or assembly of proteins, with which the sulfydryl group of the free A-chain must couple to trigger the membrane translocation process.

Two $Thy_{1.1}$-expressing murine lymphoma cell lines, AKR-A and BW5147, were to be found between 10^4 and 10^6 times less sensitive to the disulfide linked anti-$Thy_{1.1}$-gelonin conjugates than were the splenic T-cells (Thorpe et al. 1981b). This result has since been confirmed using the conjugates prepared with the chlorambucil derivative. Nevertheless, when injected at dose levels corresponding to less than 1/50 of the minimal lethal dose for free gelonin, the anti-$Thy_{1.1}$-gelonin conjugate (M, 180,000) prolonged by 1 week the median survival time of CBA mice carrying an intraperitoneal AKR-A tumor allograft (Fig. 12).

The relative resistance of the lymphoma cells to the conjugates may be connected with the fact that they are a proliferating cell population, as opposed to the T-lymphocytes which were mainly resting. In support of this suggestion, AKR T-lymphocytes which were stimulated with phytohemagglutinin or concanavalin A for 24 h before being exposed to the conjugates were as resistant as the lymphoma cells. The apparent relationship between the stage of the cell cycle and the susceptibility to the conjugate is unexplained.

iv) *Discussion:* It is evident that only certain types of cell surface receptor

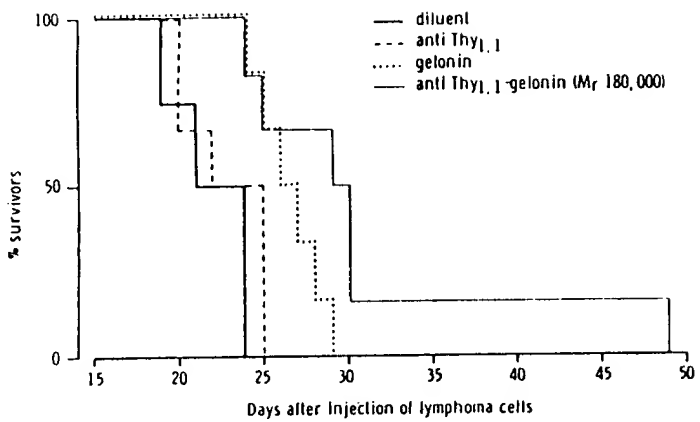


Figure 12 Prolongation of survival of immunologically-deprived CBA mice bearing a $Thy_{1.1}$-expressing lymphoma, following the administration of monoclonal anti-$Thy_{1.1}$ antibody conjugated to gelonin. The mice received an intraperitoneal injection of 10^5 AKR-A lymphoma cells and 1 day later were treated intraperitoneally with 6.7×10^{-10} moles of anti-$Thy_{1.1}$ antibody alone (- - -), gelonin (· · · · ·) or the anti-$Thy_{1.1}$-gelonin conjugate (M_r 180,000) (—)

molecules to which conjugates containing toxin A-chains bind can mediate the translocation of the A-chain across cell membranes with an efficiency which approaches that seen with the native toxin.

Conjugates of ricin A-chain with the lectins, concanavalin A (Yamaguchi et al. 1979) and that from *Wisteria floribunda* (Uchida et al. 1980), although highly effective as cytotoxic agents, recognize sugar sequences which are common to many cell surface molecules, and, of those, only a minority may be effective in mediating the penetration of the A-chain. It is difficult, therefore, to use lectin conjugates to elucidate the process by which toxin A-chains traverse cell membranes. Likewise, it is hard to analyze the mechanism by which Jansen's anti-DNP conjugates exercise their marked cytotoxicity since the conjugates would interact with a multitude of different haptenated cell surface proteins.

Ricin A-chain linked to epidermal growth factor (Cawley et al. 1980b) and both diphtheria toxin A-chain and ricin A-chain when linked to asialofetuin (Cawley et al. 1980a, 1981) were exceedingly potent cytotoxic agents. Asialofetuin and epidermal growth factor, after interacting with their receptors on hepatocytes and fibroblasts, respectively, are internalized into coated vesicles (Maxfield et al. 1978, Gorden et al. 1978, Wall et al. 1980) and this is likely also to be true for their conjugates, suggesting that receptor-mediated endocytosis may

be a route by which A-chains can escape in undegraded form into the cytoplasm. The experiments of Hubbard et al. (1979) and Wall et al. (1980) have shown that asialoorosomucoid, which is similar to asialofetuin in that it is a galactose-terminated glycoprotein, is also internalized by hepatocytes into coated vesicles. Within 30 min, more than half the internalized ^{125}I-labeled protein had become associated with the lysosomes, whereas the remainder was retained by undefined structures in the cytosol. It was suggested by Cawley et al. (1981) that the cytotoxicity of their asialofetuin-diphtheria A-chain conjugate may be due to a component which, likewise, had escaped lysosomal degradation.

The Thy_1 antigen, a non-transmembrane protein, seems to be excluded from coated pits, even when cross-linked with IgG anti-Thy_1 antibody (Bretscher et al. 1980), and yet the conjugates of anti-$Thy_{1.1}$ antibody and gelonin (Thorpe et al. 1981b) and anti-$Thy_{1.2}$ antibody and ricin A-chain (Blythman et al. 1981) were outstandingly cytotoxic. It may be that these conjugates act by a mechanism totally unrelated to that described for the asialofetuin and EGF conjugates described above, perhaps even by penetrating the plasma membrane itself. It should be noted, however, that Blythman and her colleagues employed an IgM monoclonal antibody, which potentially could form aggregates by cross-linking Thy_1 antigens upon the cell surface and that Thorpe and coworkers found that a conjugate containing aggregated IgG anti-$Thy_{1.1}$ antibody and gelonin was more than 100 times more potent than a simple conjugate with one molecule each of antibody and gelonin. It is possible that formation of such aggregates facilitates the endocytosis of the conjugate. Nevertheless, it is clear that endocytosis *per se* does not lead to the effective delivery of ricin A-chain, as was shown for the W3/25-ricin A-chain conjugate described above, and the exact pathway of internalization seems to be of paramount importance.

Epidermal growth factor, *Wisteria floribunda* lectin and anti-Thy_1 antibodies are not indiscriminate in their abilities to mediate the translocation across cell membrane of macromolecules to which they are covalently linked. Diphtheria toxin A-chain linked by a disulfide bond to these carriers was either non-toxic or only weakly cytotoxic (Cawley et al. 1980b, Uchida et al. 1980, Thorpe & Ross present communication). Thus the successful delivery of ricin A-chain depends upon attributes absent from the A-chain of the bacterial toxin. Two explanations are tenable. It is possible that the hydrophobic regions which are situated within the A-chain of ricin (Funatsu et al. 1978) but absent from that of diphtheria toxin (Delange et al. 1979) are required for it to insert into the lipophilic core of the membrane, possibly in association with the receptor to which the carrier portion of the conjugate is linked. Alternatively, ricin A-chain and gelonin are glycosylated, whereas the A-chain of diphtheria toxin is not, suggesting that the carbohydrate molecules may provide a signal which, when recognized by a transport protein or assembly of proteins, leads to the passage of the inhibitors across cell membranes. It is interesting that asialofetuin is equally

effective at killing hepatocytes when conjugated either to diphtheria toxin or ricin A-chains (Cawley et al. 1981, 1980a, b), illuminating peculiarities in the structural requirements for the membrane translocation of macromolecules taken up by the different processes.

The majority of the conjugates containing isolated A-chains which have been listed in the preceding sections did display specific cytotoxic effects upon the cells to which they could bind, but were far less potent than the native toxin. The B-chains of the toxins, in addition to providing the cell binding function, seem also to be required in later stages of the cytotoxic process. It has been suggested that ricin, following binding to a galactose-containing molecule at the cell surface, is rapidly internalized by receptor-mediated endocytosis (Nicolson 1974, 1975) and this may be necessary for expression of its cytotoxic action, in accordance with the high potency of the hybrid molecules which utilize similar uptake mechanisms. Most monoclonal antibodies of the IgG class do not induce endocytosis by capping, so that it is to be expected that their conjugates likewise will be internalized only slowly by absorptive pinocytosis. This agrees with the experimental evidence from Jansen and coworkers that their monoclonal antibody-ricin-A-chain conjugates take many hours to achieve their maximal cytotoxic effect (Jansen et al., personal communication). The widely ranging potencies of different conjugates may reflect the ease with which the cell surface antigenic molecules facilitate endocytosis and the pathway of internalization. An attractive possibility for improving the potency of an antibody-A-chain conjugate which, by itself, is weakly or non-cytotoxic, is to couple it to a moiety which can utilize a receptor-mediated endocytic pathway that efficiently permits the penetration of the A-chain. As suggested by Martinez et al. (1981), the coupling could be achieved by the use of appropriate second layer reagents reactive both with the primary conjugate and with the receptors for epidermal growth factor, low density lipoprotein etc.

4. *Conclusion*

The results obtained so far with conjugates of antibodies and intact toxins or their A-fragments are sufficiently promising to indicate that cell-type-specific toxicity can be achieved by these means. Presently, conjugates with intact toxins, although very potent, have a strong component of non-specific toxicity whereas conjugates with isolated A-chains, although specific, often lack potency. These problems should be resolved by detailed studies of methods for blocking the cell-binding site of intact toxins and of the translocation mechanisms responsible for internalizing toxin A-chains after the conjugate has attached to antigens upon the cell surface. In view of the variation in effectiveness of conjugates prepared by different methods there is much scope for examining further the influence of the linkage upon the stability and cytotoxic performance of the conjugate.

IV. SUMMARY

Antibodies have been covalently linked to intact toxins or their A-fragments by methods which neither damage the antigen-binding properties of the antibody nor the capacity of the A-chain of the toxin to inhibit protein synthesis by eukaryotic ribosomes.

Conjugates with intact diphtheria toxin, abrin or ricin display highly potent but only moderately specific cytotoxic actions upon cells in tissue culture which bear the appropriate antigens. The non-specific toxicity of the conjugates is attributable to the ability of the toxin moiety to bind to receptors which are to be found upon most cell surfaces. The conjugates are therefore highly poisonous to animals and show little evidence *in vivo* of selective cytotoxicity for cancer cells for which the antibody moiety is specific. In tissue culture the non-specific cytotoxic effects of conjugates containing abrin and ricin can be reduced by incorporating galactose in the cultures to antagonize competitively the binding of the toxins to their galactose-containing receptor molecules.

Abrin, when treated with reagents which modify primary amino groups, exhibits a reduced capacity to bind to galactose and is therefore much less toxic. The linkage of the modified toxin to monoclonal anti-$Thy_{1.1}$ antibody restores the capacity to bind to and kill $Thy_{1.1}$-expressing T-lymphocytes in tissue culture. For reasons which are obscure, the conjugate still retains non-specific toxicity for B-lymphocytes.

The A-chain of diphtheria toxin or ricin when linked by a disulfide bond to monoclonal anti-$Thy_{1.1}$ or W3/25 antibodies, respectively, is either weakly or non-cytotoxic to mouse or rat T-lymphocytes in tissue culture. Even when endocytosis of the W3/25-ricin A-chain conjugate by rat T-lymphocytes is stimulated with rabbit anti-mouse IgG no cytotoxic action ensues. By contrast, gelonin, a ribosome-damaging protein analogous to the A-chains of abrin or ricin, acquires exceedingly potent and specific cytotoxicity for AKR T-lymphocytes after its linkage to anti-$Thy_{1.1}$ antibody. The anti-$Thy_{1.1}$-gelonin conjugate is, however, much less effective when used against AKR lymphoma cells or T-lymphoblasts. Nevertheless, administration of the conjugate to mice delays the growth of the lymphoma cells in the peritoneal site.

It is concluded that cell-type-specific cytotoxic agents can be prepared by the covalent linkage of antibodies to toxins or their A-chains, but that further work is needed to elucidate the mechanism by which the toxic moiety can penetrate cell membranes after attachment of the conjugate to antigens upon the cell surface.

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REFERENCES

Baenziger, J. U. & Fiete, D. (1979) Structural determinants of Ricinus communis agglutinin and toxin specificity for oligosaccharides. *J. Biol. Chem* 254, 9795–9799.

Barrett, A. J. (Ed) (1977) *Proteinases in mammalian cells and tissues* North-Holland Publ. Co., Amsterdam, New York & Oxford.

Barrett, A. J. & Heath, M. F. (1977) Lysosomal Enzymes. In: *Lysosomes: a laboratory handbook*, 2nd edn., ed. Dingle, J. T., pp. 19–145. Elsevier/North Holland Biomedical Press.

Blythman, H. E., Casellas, P., Gros, O., Gros, P., Jansen, F. K., Paolucci, F., Pau B. & Vidal, H. (1981) Immunotoxins: hybrid molecules of monoclonal antibodies and a toxin subunit specifically kill tumor cells. *Nature* 290, 145–146.

Bretscher, M. S., Thomson, J. N. & Pearse, B. M. F. (1980) Coated pits act as molecular filters. *Proc. natl. Acad. Sci. USA* 77, 4156–4159.

Carlsson, J., Drevin, D. & Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio) propionate – a new heterobifunctional reagent. *Biochem. J.* 173, 727–737.

Cawley, D. B., Herschman, H. R., Gilliland, D. G. & Collier, R. J. (1980b) Epidermal growth factor-toxin A chain conjugates: EGF-Ricin A is a potent toxin while EGF-diphtheria toxin fragment A is non-toxic. *Cell.* 22, 563–570.

Cawley, D. B., Simpson, D. L. & Herschman, H. R. (1980a) Toxicity of an asialofetuin-ricin A chain chimera on cultured hepatocytes is mediated by the asialoglycoprotein receptor. *Fed. Proc.* 1015, 1798.

Cawley, D. B., Simpson, D. L. & Herschman, H. R. (1981) Asialoglycoprotein receptor mediates the toxic effects of an asialofetuin-diphtheria toxin fragment A conjugate on cultured rat hepatocytes. *Proc. natl. Acad. Sci. USA* 78, 3383–3387.

Chang, T., Dazord, A. & Neville, D. M. (1977) Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulphide conjugate. II Biochemical and biological properties of diphtheria toxin fragment A-SS-human placental lactogen. *J. Biol. Chem.* 252, 1515–1522.

Collier, R. J. (1976) Inhibition of protein synthesis by exotoxins from Corynebacterium diphtheriae and Pseudomonas aeruginosa. In: *Receptors and Recognition* Series B, The specificity and action of animal, bacterial and plant toxins, ed. Cuatrecasas, P., pp. 67–98. Chapman & Hall, London.

Delange, R. J., Williams, L. C., Drazin, R. E. & Collier, R. J. (1979) The amino acid sequence of fragment A, an enzymically active fragment of diphtheria toxin. *J. Biol. Chem* 254, 5838–5842.

Draper, R. K. & Simon, M. I. (1980) The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. *J. Cell. Biol.* 87, 849–854.

Edwards, D. C., Brown, A., Cumber, A. J., Smith, A., Ross, W. C. J., Thorpe, P. E. & Davies, A. J. S. (1981b) *In vitro* and *in vivo* effects of anti-mouse lymphocyte globulin, abrin and their conjugates. (submitted for publication).

Edwards, D. C., Ross, W. C. J., Cumber, A. J., McIntosh, D., Smith, A., Thorpe, P. E., Brown, A., Williams, R. H. & Davies, A. J. S. (1981c) A comparison of the *in vitro* and *in vivo* activities of conjugates of anti-mouse lymphocyte globulin and abrin prepared by the use of either N-hydroxy succinimidyl ester of chlorambucil or N-succinimidyl 3-(2-pyridyldithio)propionate (submitted for publication)

Edwards, D. C., Smith, A., Ross, W. C. J., Cumber, A. J., Thorpe, P. E. & Davies, A. J. S. (1981a) The effect of abrin, anti-lymphocyte globulin and their conjugates on the immune response of mice to sheep red blood cells. *Experientia* 37, 256–257.

Eiklid, K., Olsnes, S. & Pihl, A. (1980) Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells. *Exp. Cell. Res.* 126, 321–326.

Fritsche, R. & Mach, J. P. (1977) Isolation and characterization of carcinoembryonic antigen (CEA) extracted from normal human colonic mucosa. *Immunochemistry* 14, 197–204.

Funatsu, G., Yoshitake, S. & Funatsu, M. (1978) Primary structure of ile chain of ricin D. *Agric. Biol. Chem. (Tokyo)* 42, 501–503.

Gasperi-Campani, A., Barbieri, L., Morelli, P. & Stirpe, F. (1980) Seed extracts inhibiting protein synthesis *in vitro*. *Biochem. J.* 186, 439–441.

Ghose, T., Norvell, S. T., Guclu, A., Cameron, D., Bodurma, A. & MacDonald, A. S. (1972) Immunochemotherapy of cancer with chlorambucil-carrying antibody *Brit. med. J.* 3, 495–499.

Gill, D. M. (1978) Seven toxic proteins that cross the cell membrane. In: *Bacterial Toxins and Cell Membranes*, eds. Jeljaszewicz, J. & Wadstrom, T., pp. 291–332 Academic Press, London & New York.

Gilliland, D. G. & Collier, R. J. (1980) A model system involving anti-concanavalin A for antibody targeting of diphtheria toxin fragment A. *Cancer Res.* 40, 3564–3569

Gilliland, D. G., Collier, R. J., Mochring, J. M. & Mochring, T. J. (1978) Chimeric toxins: Toxic, disulphide-linked conjugate of concanavalin A with fragment A from diphtheria toxin. *Proc. natl. Acad. Sci. USA* 75, 5319–5323.

Gilliland, D. G., Steplewski, Z., Collier, R. J., Mitchell, K. F., Chang, T. H. & Koprowski, H. (1980). Antibody-directed cytotoxic agents: Use of monoclonal antibody to direct the action of toxin A-chains to colorectal carcinoma cells. *Proc. natl. Acad. Sci. USA* 77, 4539–4543.

Gold, P. & Freedman, S. O. (1965) Specific carcinoembryonic antigens of the human digestive system. *J. exp. Med.* 122, 467–481.

Gordeń, P., Carpentier, J. L., Cohen, S. & Orci, L. (1978) Epidermal growth factor: morphological demonstration of binding internalization, and lysosomal association in human fibroblasts. *Proc. natl. Acad. Sci. USA* 75, 5025–5029.

Habeeb, A. F. S. A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulphonic acid. *Anal. Biochem.* 14, 328–336.

Hubbard, A. L., Wilson, G., Ashwell, G. & Stukenbrok, H. (1979) An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. II Intracellular fates of the ^{125}I-ligands. *J. Cell. Biol.* 83, 65–81.

Jansen, F. K., Blythman, H. E., Carriere, D., Casellas, P., Diaz, J., Gros, P., Hennequin, J. R., Paolucci, F., Pau, B., Poncelet, P., Richer, G., Sallu, S. L., Vidal, H. & Voisin, G. A. (1980) High specific cytotoxicity of antibody-toxin hybrid molecules (immunotoxins) for target cells. *Immunol. Letters* 2, 97–102.

Kim, K. & Groman, N. B. (1965) *In vitro* inhibition of diphtheria toxin action by ammonium salts and amines. *J. Bacteriol.* 90, 1552–1556.

Krolick, K. A., Villemez, C., Isakson, P., Uhr, J. W. & Vitetta, E. S. (1980) Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin *Proc. natl. Acad. Sci. USA* 77, 5419–5423.

Martinez, O., Kimura, J., Zeicher, M. & Wolfsy, L. (1981) Effectiveness of antibody-toxin A conjugates as selective cytotoxic agents. (submitted for publication).

Mason, D. W., Brideau, R. J., McMaster, W. R., Webb, M., White, R. A. H. & Williams, A. F. (1980) Monoclonal antibodies that define T-lymphocyte subsets in the rat. In: *Monoclonal Antibodies*. eds. Kennett, R. H., McKearn, T. J. & Bechtol, K. B., pp. 251–273. Plenum Publ. Co., New York & London.

Mason, D. W. & Williams, A. F. (1980) The kinetics of antibody binding to membrane antigens in solution and at the cell surface. *Biochem. J.* **187**, 1–20.

Masuho, Y. & Hara, T. (1980) Target-cell cytotoxicity of a hybrid of Fab' of immunoglobulin and A-chain of ricin. *Gann* **71**, 759–765.

Masuho, Y., Hara, T. & Teruhisa, N. (1979) Preparation of a hybrid of fragment Fab' of antibody and fragment A of diphtheria toxin and its cytotoxicity. *Biochem. Biophys. Res. Commun.* **90**, 320–326.

Maxfield, F. R., Schlessinger, J., Shechter, Y., Pastan, I. & Willingham, M. C. (1978) Collection of insulin, EGF and α_2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. *Cell* **14**, 805–810.

Miskimins, W. K. & Shimizu, N. (1979) Synthesis of a cytotoxic insulin cross-linked to diphtheria toxin fragment A capable of recognising insulin receptors. *Biochem. Biophys. Res. Commun.* **91**, 143–151.

Miyazaki, H., Beppu, M., Terao, T. & Osawa, T. (1980) Preparation of antibody (IgG)-ricin A-chain conjugate and its biologic activity. *Gann* **71**, 766–774.

Moolten, F. L., Capparell, N. J. & Cooperband, S. R. (1972) Antitumour effects of antibody-diphtheria toxin conjugates: use of hapten-coated tumour cells as an antigenic target. *J. natl. Cancer Inst.* **49**, 1057–1062.

Moolten, F. L., Capparell, N. J., Zajdel, S. H. & Cooperband, S. R. (1975) Antitumour effects of antibody-diphtheria toxin conjugates. II Immunotherapy with conjugates directed against tumour antigens induced by simian virus. *J. natl. Cancer Inst.* **55**, 473–477.

Moolten, F. L. & Cooperband, S. R. (1970) Selective destruction of target cells by diphtheria toxin conjugated to antibody directed against antigens on the cells. *Science* **169**, 68–70.

Nicolson, G. L. (1974) Ultrastructural analysis of toxin binding and entry into mammalian cells. *Nature* **251**, 628–630.

Nicolson, G. L. (1975) Dynamic changes on the surfaces of normal and transformed cells and their relationship to the entry of pharmacologically active proteins. *Amer. J. Clin. Pathol.* **63**, 677–684.

Oeltmann, T. N. & Heath, E. C. (1979) A hybrid protein containing the toxic subunit of ricin and the cell-specific subunit of human chorionic gonadotrophin. II Biological properties. *J. Biol. Chem.* **254**, 1028–1032.

Olsnes, S. & Pihl, A. (1976) Abrin, ricin and their associated agglutinins. In: *Receptors and Recognition* Series B: The specificity and action of animal, bacterial and plant toxins, ed. Cuatrecasas, P., pp. 129–173. Chapman & Hall, London.

Olsnes, S. & Pihl, A. (1981) Chimaeric Toxins In: *Pharmacology of Bacterial Toxins*. eds. Drew, J. & Dorner, F. Pergamon Press (in press).

Olsnes, S., Saltvedt, E. & Pihl, A. (1974) Isolation and comparison of galactose-binding lectins from *Abrus precatorius* and *Ricinus communis*. *J. Biol. Chem.* **249**, 803–810.

Olsnes, S., Sandvig, K., Refsnes, K. & Pihl, A. (1976) Rates of different steps involved in the inhibition of protein synthesis by the toxic lectins abrin and ricin. *J. Biol. Chem.* **257**, 3985–3992.

Pappenheimer, A. M. Jr. (1977) Diphtheria toxin. *Ann. Rev. Biochem.* **46**, 69–94.

Raso, V. & Griffin, T. (1980) Specific cytotoxicity of a human immunoglobulin-directed Fab'-ricin A-chain conjugate. *J. Immunol.* **125**, 2610–2616.

Rector, E. S., Schwenk, R. J., Tse, K. S. & Sehon, A. H. (1978) A method for the preparation of protein-protein conjugates of predetermined composition. *J. Immunol. Methods* **24**, 321–336.

Ross, W. C. J. (1949) Aryl-2-halogenalkylamines. Part IV. The reactions of NN-di-2-chloroethyl-*p*-anisidine and β-naphthyl di-2-chloroethylamine in aqueous acetone solutions of amines. *J. Chem. Soc.* 2824–2831.

Ross, W. C. J. (1974) The interaction of chlorambucil with human γ-globulin. *Chem Biol. Interactions* **8**, 261–267.

Ross, W. C. J. (1975) The conjugation of chlorambucil with human γ-globulin. *Chem Biol. Interactions* **10**, 169–172.

Ross, W. C. J., Thorpe, P. E., Cumber, A. J., Edwards, D. C., Hinson, C. A. & Davies, A. J. S. (1980) Increased toxicity of diphtheria toxin for human lymphoblastoid cells following covalent linkage to anti-(human lymphocyte) globulin or its F(ab')$_2$ fragment. *Eur. J. Biochem.* **104**, 381–390.

Rubens, R. D. (1974) Antibodies as carriers of anticancer drugs. *Lancet* i, 498–499.

Sandvig, K. & Olsnes, S. (1979) Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. *Exp. Cell. Res.* **121**, 15–25.

Sandvig, K. & Olsnes, S. (1980) Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell. Biol.* **87**, 828–832.

Sandvig, K., Olsnes, S. & Pihl, A. (1976) Kinetics of binding of the toxic lectins abrin and ricin to surface receptors of human cells. *J. Biol. Chem.* **251**, 3977–3984.

Sandvig, K., Olsnes, S. & Pihl, A. (1978) Chemical modification of the toxic lectins abrin and ricin. *Eur. J. Biochem.* **84**, 323–331.

Stirpe, F., Olsnes, S. & Pihl, A. (1980) Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. Isolation, characterization and preparation of complexes with concanavalin A. *J. Biol. Chem.* **255**, 6947–6953.

Thorpe, P. E., Brown, A. N. F., Ross, W. C. J., Cumber, A. J., Detre, S. I., Edwards, D. C., Davies, A. J. S. & Stirpe, F. (1981b) Cytotoxicity acquired by conjugation of an anti-Thy$_{1.1}$ monoclonal antibody and the ribosome-inactivating protein, gelonin. *Eur. J. Biochem.* **116**, 447–454.

Thorpe, P. E., Cumber, A. J., Williams, N., Edwards, D. C., Ross, W. C. J. & Davies, A. J. S. (1981a) Abrogation of the non-specific toxicity of abrin conjugated to anti-lymphocyte globulin. *Clin. exp. Immunol.* **43**, 195–200.

Thorpe, P. E., Edwards, D. C., Ross, W. C. J. & Davies, A. J. S. (1982) Monoclonal antibody-toxin conjugates: aiming the magic bullet In: *Monoclonal antibodies in clinical medicine*. eds. Fabre, J. & McMichael, A. Academic Press, London. (in press)

Thorpe, P. E., Ross, W. C. J., Cumber, A. J., Hinson, C. A., Edwards, D. C. & Davies, A. J. S. (1978) Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to anti-lymphocyte globulin. *Nature* **271**, 752–755.

Uchida, T., Mekada, E. & Okada, Y. (1980) Hybrid toxin of the A chain of ricin and a subunit of *Wisteria floribunda* lectin. Possible importance of the hydrophobic region for entry of toxin into the cell. *J. Biol. Chem.* **255**, 6687–6693.

Uchida, T., Pappenheimer, A. M. Jr. & Greany, R. (1973) Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* **248**, 3838–3844.

Uchida, T., Yamaizumi, M., Mekada, E., Okada, Y., Tsuda, M., Kurokawa, T. & Sugino, Y. (1978) Reconstitution of hybrid toxin from fragment A of diphtheria toxin and a subunit of *Wisteria floribunda* lectin *J. Biol. Chem.* **253**, 6307–6310.

Wall, D. A., Wilson, G. & Hubbard, A. L. (1980) The galactose-specific recognition system of mammalian liver: the route of ligand internalisation in rat hepatocytes.

Cell 21, 79–93.

Yamaguchi, T., Kato, R., Beppu, M., Terao, T., Inoue, Y., Ikawa, Y. & Osawa, T. (1979) Preparation of concanavalin A-ricin A-chain conjugate and its biologic activity against various cultured cells. *J. natl. Cancer Inst.* 62, 1387–1395.

Yamazumi, M., Mekada, E., Uchida, T. & Okada, Y. (1978) One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* 15, 245–250.

Youle, R. J., Murray, G. J. & Neville, D. M. (1979) Ricin linked to monophosphopentamannose binds to fibroblast lysosomal hydrolase receptors resulting in a cell-type-specific toxin. *Proc. natl. Acad. Sci. USA* 76, 5559–5562.

Youle, R. J., Murray, G. J. & Neville, D. M. Jr. (1981) Studies on the galactose-binding site of ricin and on the hybrid toxin Man6P-ricin. *Cell* 23, 551–559.

Youle, R. J. & Neville, D. M. (1980) Anti-Thy-1 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. *Proc. natl. Acad. Sci. USA* 77, 5483–5486.

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Neoplastic B Cells as Targets for Antibody-Ricin A Chain Immunotoxins

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INTRODUCTION

The targeting of toxic agents to tumor cells *in vivo* has been a goal of immunological research since the studies of Ehrlich (Himmelweit 1960). Successful application of this technique requires tumor-specific antibody whose activity remains unaltered following its covalent conjugation to a toxic agent. Moreover, the toxic portion of the conjugate should remain inactive until bound to the tumor cell *via* its antibody portion. In the studies described in this review, we have used an antibody against the cell surface immunoglobulin idiotype (Id) of a B cell tumor as our model system. B cell tumors are virtually always monoclonal in origin; hence each tumor cell that bears surface immunoglobulin expresses a particular Id (Fu et al. 1975, Schroer et al. 1974, Salsano et al. 1974). Since the Id is present on only a very small number of normal B cells (approximately $1/10^6$), the Id is operationally a tumor-specific antigen. Other advantages of using B cell tumors as model systems for antibody targeting studies include the availability of antibodies against other determinants on the cell surface immunoglobulin molecule (isotype, allotype, etc.) and a large body of information concerning the role of particular organs (e.g., spleen), cell types (T cells, macrophages) and lymphokines on the replication and differentiation of both normal and neoplastic B cells. In addition, a large number of humans with B cell tumors do not respond well to conventional chemo/radiotherapy (Lennert & Mohri 1978) and there is, therefore, a need to improve treatment.

Recent studies utilizing antibody-directed targeting of toxic peptides to the surface of neoplastic cells are summarized in Table 1 and are reviewed in this volume. In general, the results of these studies have been promising. However,

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Myeloid-Associated Differentiation Antigens on Stem Cells and Their Progeny Identified by Monoclonal Antibodies

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Within the hematopoietic system, monoclonal antibodies reactive with antigenic determinants, expressed in a lineage- and stage-restricted fashion, can be used to map myeloid differentiation. We have generated a series of monoclonal antibodies that reacts with myeloid-associated determinants on committed myeloid stem cells and their progeny. Their reactivity with peripheral blood cells was identified by immunofluorescence assays, with bone marrow cells by fluorescence-activated cell sorting, and with committed hematopoietic progenitor cells by both cytotoxic assays and fluorescence-activated cell sorting. Antibody 1G10, which has previously been reported to react with cells of the granulocytic lineage and with a minor subset of mature monocytes, was shown to react with granulocyte-macrophage colony-forming units (CFU-GM). Three antibodies not previously characterized (T5A7, L4F3, L1B2) were shown to react with both granulocytic and monocytic cells and in fluorescence-activated cell sorting studies to detectably stain granulocytic cells at different stages of maturation. These three antibodies also react with CFU-GM, two (L4F3 and L1B2) reacting with all CFU-GM, while T5A7 reacts with only a portion of the day 7 CFU-GM. Antibody L4F3 also reacts with a portion of erythroid burst-forming units (BFU-E). In contrast, the previously reported antibody 5F1, which reacts with monocytic cells, nucleated erythroid cells, and platelets, was shown to react with erythroid colony-forming units (CFU-E). Potential applications of these antibodies to studies of normal and malignant hematopoiesis are discussed.

SPECIFIC MORPHOLOGICAL and histochemical changes serve to define stages of differentiation during terminal myeloid maturation. In addition, cell surface changes, which include the development of complement receptors,[1,2] receptors for the Fc region of immunoglobulin,[3,4] and maturation-linked loss of histocompatibility antigens,[5,6] have been described. However, none of these surface markers allows discrimination between lineages of hematopoietic cells, nor do they distinguish between stem cells of different lineages.

The development of hybridoma technology[7] has allowed generation of monoclonal antibodies selectively reactive with antigenic determinants expressed in the hematopoietic system by myeloid cells at specific stages of differentiation.[8-22] Such antibodies are useful for examining cell surface alterations during myeloid differentiation and are potentially useful for isolating and further characterizing subpopulations of cells at specific stages of maturation, including stem cells. Recently, Linker-Israeli et al.[13] and Hanjan et al.[21] have described monoclonal antibodies that react with monocyte-associated determinants and, in combination with complement, can inhibit the growth of granulocyte-macrophage colony-forming units (CFU-GM). The use of antibodies reactive with myeloid-associated differentiation antigens to characterize normal and malignant hematopoietic stem cell populations, however, has not been fully exploited. For the purpose of further characterizing hematopoietic stem cells as well as myeloid differentiation, we have generated five monoclonal antibodies reactive with myeloid-associated antigenic determinants. Two of these antibodies, 1G10 and 5F1, have been reported previously.[18,19] Antibody 1G10 is reactive with a determinant expressed in high concentration by virtually all mature and immature granulocytes present in peripheral blood and bone marrow, and in low concentrations on a minor subset of monocytes. Antibody 5F1 detects a determinant expressed in peripheral blood by monocytes and platelets and in bone marrow by nucleated erythroid cells, monocytes, and megakaryocytes. Preliminary evidence indicated that treatment of bone marrow cells with antibodies 1G10 and 5F1 plus complement inhibited CFU-GM and (CFU-E), respectively, although the reactivity of these antibodies with the progenitor cells themselves was not directly demonstrated.[19] For the present studies we produced three additional antibodies—L1B2, L4F3, and T5A7—which react with granulocytic cells at different stages of differentiation and with monocytes. We describe the reactions of these antibodies with myeloid cells, including myelocytic and erythrocytic progenitors.

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MATERIALS AND METHODS

Production of Monoclonal Antibodies

Leukemic cells from each of three patients with acute nonlymphoblastic leukemia (ANL) were used as immunogens for three separate fusions. Details of the immunization schedules, fusion methods,

and the initial culture and screening of hybrids have been previously described.[18] Spleen cells from immunized mice were fused with the P3-NS1-1-Ag4-1 myeloma cell line. Hybrids that secreted antibody cytotoxic for the immunizing cell type but not for a B-cell line derived from one of the patients were formally cloned twice by the technique of limiting dilution. The resultant clones were grown as ascites tumors in pristane-primed BALB/c mice. Unless specified, all antibodies used in the studies presented here were in the form of unfractionated ascites fluids.

Determination of Monoclonal Antibody Isotype

The isotype of monoclonal antibody present in ascites fluids was determined by immunoprecipitation using immunodiffusion plates (Hyland Diagnostics, Division of Travenol Laboratories, Deerfield, IL) with rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM antisera (Litton Bionetics, Kensington, MD).

Peripheral Blood and Bone Marrow Cells

Samples of peripheral blood and bone marrow were obtained from normal healthy volunteers following informed consent. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque density (specific gravity 1.077) gradient centrifugation. Bone marrow cells used for culture experiments were separated by diluting the sample 1:1 with RPMI 1640 (Grand Island Biological Co., Grand Island, NY) in a 15-ml conical bottom test tube (Falcon, Division of Becton Dickinson, Oxnard, CA) and centrifuging at 1,000 × *g* for 10 min. Buffy coat cells were removed and resuspended in RPMI 1640. Following 3 sequential buffy coats, residual red cells were lysed using a 0.155 *M* ammonium chloride buffer.

Bone marrow cells used for analysis and separation by fluorescence-activated cell sorting were isolated by using a specific gravity 1.108 Ficoll-Hypaque cushion for density gradient separation.

Samples of thymocytes, tonsil cells, and spleen cells were kindly provided by Dr. P. Martin, Fred Hutchinson Cancer Research Center, Seattle, WA.

Peripheral Blood Granulocytes

Peripheral blood granulocytes were isolated by a two-step Ficoll-Hypaque density gradient in which 4 ml of Ficoll-Hypaque (specific gravity 1.108) was overlaid in a 15-ml polystyrene conical bottom test tube with 2 ml of Ficoll-Hypaque (specific gravity 1.090), and 5 ml of heparinized blood was then layered on the gradient. The sample was centrifuged at 350 × *g* for 30 min. Mononuclear cells were separated at the top of the 1.090 specific gravity layer and granulocytes were isolated as a broad band within the 1.108 layer. Cells isolated from the 1.108 specific gravity layer were >98% viable by trypan blue dye exclusion and >99% granulocytes as determined by cytocentrifuge preparations stained with Wright-Giemsa and peroxidase stains.

Adherent and Nonadherent Cell Separations

Peripheral blood adherent and nonadherent cells were separated by means of plastic adherence as previously described.[18] Adherent cells thus obtained were >95% viable by trypan blue exclusion. Cytocentrifuge slide preparations stained with Wright-Giemsa and alpha-naphthylacetate esterase (NSE) showed the adherent cells to be 85%–95% monocytes. The nonadherent cell population was >98% viable by trypan blue exclusion and <4% monocytes as determined by Wright-Giemsa and NSE stains. To enrich for B cells, T cells were depleted from the nonadherent lymphocytes by rosetting with S-2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells.

Phytohemagglutinin-Stimulated Lymphocytes

Nonadherent peripheral blood mononuclear cells were cultured at 10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS) in the presence of 3 μg/ml of phytohemagglutinin (PHA) (Burroughs-Wellcome, Research Triangle Park, NC), or without PHA, for 72–96 hr at 37°C, 5% CO_2 in air. Resultant cells were harvested and examined for reactivity with antibodies by indirect immunofluorescent techniques.

Immunofluorescent Studies

Cells were labeled using a $1:10^3$ dilution of primary antibody (ascites fluid), followed by a second step of a 1:40 dilution of an affinity-purified fluorescein-conjugated goat anti-mouse IgM or IgG antiserum (Tago, Inc., Burlingame, CA) as previously described.[18] Fluorescein-conjugated antibodies were centrifuged at $10^5 \times g$ for 10 min immediately prior to use in all assays. Antibodies used as negative controls for immunofluorescent studies were murine monoclonal antibodies of the same isotype as the test antibodies (IgG_{2a}, 19E12; IgM, T11D7 was provided by Dr. Ed Clark, University of Washington, Seattle, WA) but with extraneous specificity (both are anti-mouse Thy-1.1). Stained cells were analyzed by flow microfluorimetry using a FACS II (Becton Dickinson, Oxnard, CA) at a rate of 10^3/sec and examined by phase contrast and fluorescence microscopy.

Nucleated bone marrow cells thus analyzed were separated into fluorescent and nonfluorescent populations by fluorescence-activated cell sorting. Separated cells were maintained on ice until examined by culturing them in vitro or by preparing cytocentrifuge slides, which were stained with Wright-Giemsa stain and for peroxidase, alpha-naphthylacetate esterase, and AS-D chloroacetate esterase activity (Sigma Histozyme Kits, Sigma Chemical Co., St. Louis, MO).

Antibody-Dependent Complement-Mediated Cytolysis

A quantity of 2.0×10^6 bone marrow buffy coat cells were incubated in 200 μl of a $1:10^3$ dilution of antibody in RPMI 1640 supplemented with 20% FCS for 30 min at room temperature. Two hundred microliters of a 1:2 dilution of prescreened rabbit serum was added and incubation continued for 60 min at room temperature. Controls were treated with the monoclonal IgM antibody T11D7 (anti-mouse Thy-1.1) and complement. Samples were washed 3 times with RPMI 1640 and suspended in Alpha-plus medium (Alpha modification of Eagles minimal essential medium, Flow Laboratories, Inglewood, CA; supplemented with nucleotides and deoxyribonucleotides) at a cell concentration of 10^6/ml based on the number of cells present in the sample prior to antibody treatment.

Serum obtained from 17–21-day-old New Zealand white rabbits (R&R Rabbitries, Everett, WA) of either sex was used as a source of complement. Serum was screened for lack of nonspecific toxicity as well as for its ability to mediate specific toxicity against committed myelocytic and erythrocytic progenitors cultured in vitro. Unless otherwise specified, rabbit serum was used at a final concentration of 1:4.

In Vitro Cultures

Granulocyte-macrophage colony-forming cells (CFU-GM) were cultured in semisolid agar medium consisting of Alpha-plus medium, 0.3% agar (Difco Laboratories, Detroit, MI), 20% FCS (Sterile Systems Inc., Logan, UT), 100 U/ml penicillin G, and 100 μg/ml streptomycin (M.A. Bioproducts, Walkersville, MD) with unfractionated human placenta conditioned medium, prepared according to the methods of Schlunk and Schleyer,[23] added as a source of colony-stimulating activity. Bone marrow cells were cul-

tured at 1–2 × 10^5/ml in 35-mm tissue culture dishes (Lux Scientific, Newbury Park, CA). Cultures were incubated at 37°C, 5% CO_2, 100% humidity. Colonies containing ≥ 50 cells were scored at days 7 and 14.

Erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E) were grown in a plasma clot culture system consisting of Alpha-plus medium, 30% FCS, 10% bovine serum albumin (Fraction V, Sigma Chemical Co.), 10% beef embryo extract (Grand Island Biological Co.), 10^{-4} *M* beta-mercaptoethanol, 70 μg/ml $CaCl_2$, 100 U/ml penicillin G, 100 μg/ml streptomycin, 10% citrated bovine plasma (Colorado Serum, Denver, CO), and erythropoietin (sheep fraction III, Connaught Laboratories, Swift Water, PA); 0.5 IU/ml for CFU-E and 2.0 IU/ml for BFU-E. Cells were cultured at 1–2 × 10^5/ml. Cultures were plated as 150-μl aliquots in wells of flexible microtiter plates (Dynatech Laboratories, Alexandria, VA) and incubated at 37°C, 5% CO_2, 100% humidity. CFU-E were harvested at day 7 and BFU-E at day 14. Clots were dried and fixed with 5% glutaraldehyde onto microscope slides and then stained to identify hemoglobin-containing cells.[24]

RESULTS

Five monoclonal antibodies reactive with granulocytic and monocytic cells in peripheral blood or bone marrow were generated using cells from patients with acute nonlymphoblastic leukemia (ANL) as the immunogens. Each antibody mediates complement-dependent lysis and is of the IgM isotype, except for L1B2, which is an IgG_{2a} antibody. The reactions of these antibodies with peripheral blood and bone marrow elements and committed hematopoietic progenitors were examined in the following studies. The previously reported reactivities of antibodies 5F1 and 1G10 with peripheral blood and bone marrow[18] are included for comparative purposes.

Characterization of Antimyeloid Monoclonal Antibody Reactivity With Peripheral Blood Cells

Indirect immunofluorescent tests were performed with separated granulocyte, adherent monocyte, and nonadherent lymphocyte populations from six healthy individuals. Each of the five antibodies, except for L1B2, reacted with peripheral blood elements.

When granulocytes were tested, three antibodies were found to react with virtually all the cells, two of which reacted strongly (1G10, T5A7) and one weakly (L4F3) (Fig. 1). When adherent monocytes were tested, most cells were stained by three of the antibodies, with 5F1 and T5A7 binding in relatively higher amounts and L4F3 in a relatively lower amount. The previously described low level of reactivity of 1G10 with a minor subset of monocytes was also observed.[18]

Nonadherent cells, consisting mainly of lymphocytes, were nonreactive with each of the antibodies (Fig. 1), as were B-cell-enriched nonadherent lymphocytes, tonsil cells, spleen cells, erythrocytes, and thymocytes (data not shown). One of the antibodies, T5A7, reacted with approximately 30% of PHA-stimulated lymphocytes containing greater than 90% T cells, as determined by staining with a pan-T-cell reactive antibody, 10.2 (data not shown); 5F1 reacted with platelets. Thus, we have identified four determinants selectively expressed in peripheral blood by cells of granulocytic and/or monocytic origin.

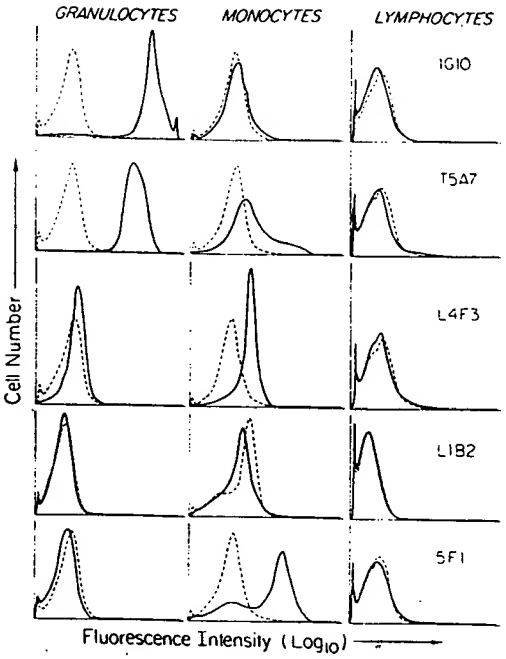


Fig. 1. Reactivity of monoclonal antibodies with peripheral blood granulocytes, adherent monocytes, and nonadherent lymphocytes: FACS analysis. Purified populations of granulocytes, adherent monocytes, and nonadherent lymphocytes were stained by indirect immunofluorescent technique as described, using each monoclonal antibody at a dilution of 1:10^3. Control samples were stained with antibodies of the same isotype and irrelevant specificity. For analysis of L1B2 reactivity, the negative control antibody was a murine monoclonal IgG_{2a} (19E12, anti-mouse Thy-1.1), and for all other antibodies, the negative control was a murine monoclonal IgM (T11D7, anti-mouse Thy-1.1). Samples were analyzed using a FACS-II equipped with a four-decade logarithmic amplifier, with photomultiplier setting of 850 V and a 546-nm green wide-band filter in place to decrease background autofluorescence. Histograms from a representative experiment show analysis of 10^4 cells in each sample. Control (-----); antibody (——).

Characterization of Monoclonal Antibody Reactivity With Normal Bone Marrow

Individual cell populations in bone marrow reactive with the monoclonal antibodies were separated into positively and negatively staining cell populations by fluorescence-activated cell sorting. For the purposes of sorting, cells were considered positively stained if they had a level of fluorescence greater than that of the negative control population. Cytocentrifuge slides were prepared from the isolated populations and stained with Wright-Giemsa, peroxidase, and AS-D chloroacetate esterase (to identify granulocytes) or NSE stains (to identify monocytes).

Table 1. Reactivity of Monoclonal Antibodies With Bone Marrow Cells Examined by Indirect Immunofluorescent Staining and Fluorescence-Activated Cell Sorting

	1G10			T5A7				L4F3			L1B2			5F1		
Cell Type*	Unsep. (%)	Positive (%)†	Negative (%)	Unsep.	Positive Bright‡	Positive Dull‡	Negative	Unsep.	Positive§	Negative	Unsep.	Positive‖	Negative	Unsep.	Positive¶	Negative
Granulocytic																
Myeloblast	0.8 ± 0.4	0.7 ± 0.7	0.7 ± 0.3	0.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.3	0.7 ± 0.4	2.0 ± 0.6	0.7 ± 0.7	2.0 ± 2.0	1.0 ± 1.0	0.0 ± 0.0	1.2 ± 0.2	0.0 ± 0.0	0.7 ± 0.6
Promyelocyte	1.3 ± 0.4	3.7 ± 1.5	0.5 ± 0.3	1.5 ± 0.3	0.0 ± 0.0	1.8 ± 0.5	1.0 ± 0.4	1.3 ± 0.3	3.3 ± 2.3	0.0 ± 0.0	1.0 ± 1.0	2.5 ± 0.5	0.0 ± 0.0	1.6 ± 0.3	0.0 ± 0.0	1.0 ± 0.6
Myelocyte	5.7 ± 0.3	12.3 ± 1.7	0.5 ± 0.3	11.3 ± 1.9	0.0 ± 0.0	3.5 ± 1.5	21.8 ± 4.3	10.0 ± 2.1	21.0 ± 9.5	0.0 ± 0.0	3.5 ± 0.5	61.5 ± 10.5	0.0 ± 0.0	5.0 ± 1.0	1.5 ± 0.8	2.8 ± 1.5
Metamyelocyte	22.0 ± 4.6	40.7 ± 5.6	5.7 ± 2.0	19.5 ± 2.7	0.5 ± 0.3	33.8 ± 5.0	41.0 ± 6.3	17.7 ± 2.7	45.0 ± 0.0	6.7 ± 2.7	19.5 ± 6.5	21.5 ± 8.5	15.5 ± 7.5	15.0 ± 2.1	2.7 ± 0.9	31.0 ± 1.2
Band	23.3 ± 3.8	18.3 ± 4.4	1.5 ± 1.3	20.5 ± 2.4	41.0 ± 2.3	48.0 ± 6.3	2.5 ± 0.7	18.3 ± 1.4	14.6 ± 5.8	30.7 ± 6.6	26.0 ± 1.0	3.0 ± 2.0	21.0 ± 5.0	27.3 ± 5.9	3.0 ± 0.6	23.7 ± 3.8
PMN	20.7 ± 5.4	21.0 ± 7.8	1.7 ± 0.3	19.3 ± 1.0	53.0 ± 3.7	1.8 ± 0.3	0.0 ± 0.0	19.3 ± 1.3	6.0 ± 2.7	28.0 ± 2.1	26.0 ± 1.0	1.0 ± 0.5	28.5 ± 1.5	15.7 ± 1.2	7.0 ± 1.5	14.3 ± 2.2
Monocytic	3.5 ± 1.8	1.0 ± 0.5	19.3 ± 4.3	5.0 ± 1.3	4.8 ± 1.5	6.0 ± 2.8	1.3 ± 0.3	4.8 ± 1.5	4.0 ± 1.5	0.5 ± 1.2	4.0 ± 3.0	6.0 ± 0.5	5.0 ± 0.5	5.0 ± 1.0	17.7 ± 3.8	0.0 ± 0.0
Lymphocytic	6.0 ± 1.7	0.3 ± 0.2	27.0 ± 7.0	5.3 ± 1.3	0.5 ± 0.5	1.8 ± 0.7	17.5 ± 7.1	5.3 ± 1.2	0.5 ± 0.3	24.0 ± 10.4	8.5 ± 2.5	0.0 ± 0.0	16.5 ± 2.5	9.0 ± 4.0	0.0 ± 0.0	22.3 ± 8.6
Erythrocytic	18.7 ± 5.8	0.7 ± 0.2	42.3 ± 9.3	17.5 ± 5.2	0.0 ± 0.0	1.5 ± 0.5	13.8 ± 2.3	22.0 ± 3.6	2.7 ± 0.9	8.7 ± 2.3	7.0 ± 0.5	0.0 ± 0.0	13.5 ± 9.5	13.7 ± 5.6	67.3 ± 4.3	0.0 ± 0.0

Bone marrow cells were stained with monoclonal antibodies by indirect immunofluorescent technique and separated by fluorescence-activated cell sorting using a FACS-II equipped with a four-decade logarithmic amplifier, with photomultiplier setting of 850 V and a 546-nm green wide-band filter to decrease background autofluorescence. Cells with fluorescence intensity greater than that of the negative control were defined as the positively fluorescent population. Cytocentrifuge slides were prepared from unseparated, positively fluorescent and negatively fluorescent populations stained with each antibody. The solid boxes indicate where more than 60% of a cell type sorted was collected in the positive population and the dashed boxes where 25%-60% was collected.

*Percent of each cell type was determined by examining ·200 cells from each population. The results are the mean ± SEM of 3 experiments.

†1G10-positive population was 47.8% ± 8.6% of cells sorted and included ·90% of sorted granulocytes and band forms, metamyelocytes and myelocytes, ·80% of sorted promyelocytes, and ·30% of myeloblasts. Thirteen percent of sorted monocytes were contained in the positive population.

‡T5A7 bright positive was 17.5% ± 2.8% and dull positive was 9.0% ± 2.1% of cells sorted and included ·90% of sorted granulocytes and band forms and ·60% of sorted monocytes.

§L4F3-positive population was 25% ± 3.6% of cells sorted and included ·70% of sorted metamyelocytes, ·90% of sorted myelocytes and promyelocytes, 50% of sorted myeloblasts, and ·70% of sorted monocytes.

‖L1B2-positive population was 14.7% ± 5.7% of cells sorted and included 25% of sorted metamyelocytes and ·90% of sorted myelocytes, promyelocytes, and myeloblasts. Twenty-one percent of sorted monocytes were contained in the positive population.

¶5F1-positive population was 14.3% ± 3.9% of cells sorted and included ·80% of monocytes and nucleated erythroid cells sorted.

Each of the five antibodies reacted with a subset of normal bone marrow cells (Table 1). Four reacted with myelocytic elements. Antibody 1G10 labeled a subset of marrow elements which, when separated, contained a portion of the myeloblasts and greater than 90% of the myelocytic cells past the myeloblast stage in the sorted populations. Each of the other three antibodies positively stained and led to selection of subsets of myelocytic cells at different stages of maturation. T5A7 stained two discrete populations: a more highly fluorescent one consisted almost exclusively of mature granulocytes and band forms, and a moderately fluorescent one contained mainly band forms and metamyelocytes. These two populations contained virtually all of the mature granulocytic cells, including band forms present in the sorted populations. The collected populations stained with L4F3 contained the majority of metamyelocytes, myelocytes, and promyelocytes and a portion of the myeloblasts present in the sorted populations, while the L1B2-stained population contained only a portion of metamyelocytes and the majority of myelocytes, promyelocytes, and myeloblasts.

Immunofluorescent staining of greater than 20% of collected bone marrow monocytes by each of the antibodies except for 1G10 was also observed. Antibodies T5A7, L4F3, as well as 5F1, which did not react with granulocytic cells, detectably stained most monocytes, while the L1B2-stained population contained only a minor proportion of marrow monocytes. Only 5F1 reacted with other cellular elements in bone marrow, including nucleated erythroid cells and megakaryocytes. While not selected in sorting experiments, megakaryocytes were observed to be reactive by fluorescence microscopy.

Reactivity of Monoclonal Antibodies With Committed Hematopoietic Progenitor Cells

The reactivity of each antibody with committed hematopoietic precursors, including CFU-GM, CFU-E, and BFU-E, was assessed first in negative selection experiments by the reduction of colony growth after treatment of bone marrow cells with antibody in the presence of complement. Since this method may also lead to deletion of auxiliary cells required for stem cell growth, falsely positive conclusions can be reached. Therefore, in positive selection experiments, bone marrow cells reactive with antibody in indirect immunofluorescent tests were isolated by fluorescence-activated cell sorting and then cultured for colony growth.

Reactivity With Myelocytic Precursors (CFU-GM)

In negative selection experiments, four of the antibodies inhibited CFU-GM growth when bone marrow cells were treated with antibody and complement (Table 2). The growth of the more mature progenitors, which gave rise to colonies on day 7, was inhibited by these four antibodies, although T5A7 only partially inhibited colony growth. Colony formation by less mature progenitors, day-14 CFU-GM, was partially inhibited by 1G10 treatment, not inhibited by T5A7, and almost completely inhibited antibodies L4F3 and L1B2. Antibody 5F1 did not affect the growth of either day-7 or day-14 CFU-GM.

For each antibody that inhibited CFU-GM in negative selection experiments (Table 2), results were confirmed by positive selection experiments in which bone marrow cells labeled with each antibody were sepa-

Table 2. Effect of Treatment With Monoclonal Antibody Plus Complement on Committed Myelocytic Progenitors

Antibody	CFU-GM	
	Day 7	Day 14
T11D7 (control)	105.6 ± 18.8	43.6 ± 6.4
1G10	29.6 ± 7.8 (72%)*†	29.8 ± 3.5 (32%)*
T5A7	74.3 ± 10.4 (30%)*	37.3 ± 3.6 (16%)
L4F3	1.5 ± 0.8 (99%)*	3.0 ± 1.1 (93%)*
L1B2	3.5 ± 2.2 (97%)*	6.6 ± 3.1 (85%)*
5F1	88.3 ± 23.2 (16%)	52.5 ± 3.2 (0%)

Bone marrow buffy coat cells were treated with antibody and complement and cultured as described. Controls were cells treated with an IgM monoclonal antibody of irrelevant specificity (T11D7) and complement. Results are the mean ± SEM of colonies per 2×10^5 marrow cells for 3 experiments done in triplicate.

*$p < 0.01$.

†Percent inhibition of colony formation.

Table 3. Reactivity of Monoclonal Antibodies With CFU-GM Determined by Indirect Immunofluorescent Staining and Fluorescence-Activated Cell Sorting

	CFU-GM	
	Day 7	Day 14
Unseparated	56.6 ± 5.1	40.3 ± 5.7
1G10 positive	25.5 ± 5.2	11.0 ± 4.4
1G10 negative	66.3 ± 6.3	64.9 ± 4.9
Unseparated	41.9 ± 10.2	9.6 ± 3.0
T5A7 positive bright	34.6 ± 14.0	0.2 ± 0.2
T5A7 positive dull	103.0 ± 39.8	3.0 ± 1.2
T5A7 negative	52.8 ± 6.0	34.8 ± 7.6
Unseparated	48.6 ± 6.9	12.5 ± 2.4
L4F3 positive	81.3 ± 21.2	14.7 ± 8.4
L4F3 negative	2.5 ± 0.6	0.5 ± 0.3
Unseparated	48.8 ± 4.2	21.0 ± 1.6
L1B2 positive	70.8 ± 2.0	14.7 ± 3.7
L1B2 negative	51.0 ± 11.9	21.9 ± 5.4

Bone marrow cells were stained and separated by fluorescence-activated cell sorting as described (see Table 1). Cells were sorted into positive and negative populations. As a control, cells from the stained sample were run through the sorter and all cells collected (unseparated). Cells from each of the fluorescent, nonfluorescent, and unseparated populations were cultured for CFU-GM. Results are the mean ± SEM of colonies per 10^5 marrow cells for each population from 2 experiments in which each population was cultured in duplicate or triplicate.

rated into positively and negatively stained populations (Table 3). The criteria for determining positively staining cells for sorting in these experiments was the same as outlined for characterization of antibody reactivity with bone marrow. Labeling with 1G10, L4F3, or L1B2 led to the collection in the fluorescent population of both day-7 and day-14 colony-forming cells. Labeling with T5A7 led to the positive selection of only the day-7 colony-forming cells. Thus, the results of cell sorting studies strongly suggested that the reduction of colony growth by treatment with antibody plus complement was due to a direct lytic effect on those cells.

1G10 and L1B2 lysed the majority of day-7 CFU-GM colony-forming cells, but detectably stained only a portion of these. We therefore examined in the case of 1G10 whether this was due to the expression of these antigens in relatively low amounts by determining whether cells stained with 1G10 and collected in the nonfluorescent population were susceptible to lysis by 1G10 and complement. The results demonstrated that this treatment reduced the remaining colony-forming activity detectable on day 7 by 60%, suggesting that a low level of 1G10 antigen is expressed by some nonfluorescent cells (data not shown).

Reactivity With Committed Erythrocytic Progenitors

The influence of each monoclonal antibody on mature (CFU-E) and immature (BFU-E) erythrocytic progenitors was examined. When CFU-E formation was examined following treatment of marrow cells with antibody plus complement, only treatment with 5F1 reduced colony growth (Table 4). Direct evidence for expression of the 5F1 antigen on CFU-E came from positive selection experiments. In these experiments, the 5F1-positive populations were enriched 12-fold for CFU-E and contained 70%–100% of all CFU-E present in the unsorted marrow sample (Table 5).

Examination of BFU-E revealed that treatment with antibody L4F3 in combination with complement produced partial inhibition of growth, while L1B2 treatment resulted in a minor and not statistically significant effect (Table 4). The reactivity of antibodies L4F3 and L1B2 with BFU-E was therefore directly assessed by isolating cells stained with each of these antibodies. A portion of the total BFU-E present in sorted marrow was found in the L4F3-labeled population (Table 6). Presumably, insignificant activity was found in the L1B2-labeled cells.

Table 5. Reactivity of Monoclonal Antibody 5F1 With CFU-E Determined by Indirect Immunofluorescent Staining and Fluorescence-Activated Cell Sorting

	CFU-E
Unseparated	49.6 ± 7.5
5F1 positive	615.0 ± 155.8
5F1 negative	2.7 ± 2.5

Bone marrow cells were stained and separated by fluorescence-activated cell sorting as described (see Table 3). Cells from each of the sorted populations were cultured for CFU-E. Results are the mean ± SEM of colonies per 10^5 marrow cells for each population from 2 experiments, in which each population was cultured in duplicate or triplicate.

DISCUSSION

Within the hematopoietic system, monoclonal antibodies reactive with myeloid-associated antigenic determinants that are lineage- and stage-restricted in expression can be used to map myelocytic and erythrocytic differentiation. In the present study we have identified cell surface antigenic alterations occurring during the course of myeloid differentiation. Of these antigenic determinants, one (1G10) is mainly expressed by the cells of the granulocytic lineage, including CFU-GM, and by a minor subset of monocytes. Three are found on both granulocytes and monocytes, and the expression of each of these can be detected at different stages of granulocytic differentiation by indirect immunofluorescent assays. Thus, T5A7 stained virtually all of the most mature myelo-

Table 4. Effect of Treatment With Monoclonal Antibody Plus Complement on Committed Erythrocytic Progenitors

Antibody	CFU-E	BFU-E
T11D7 (control)	80.8 ± 16.0	21.8 ± 4.7
1G10	67.2 ± 19.4 (17%)*	22.5 ± 7.4 (0%)
T5A7	86.6 ± 25.0 (0%)	35.8 ± 6.3 (0%)
L4F3	64.8 ± 12.0 (20%)	11.8 ± 5.5 (46%)‡
L1B2	93.9 ± 17.0 (0%)	16.5 ± 4.5 (24%)
5F1	18.9 ± 9.0 (77%)†	22.5 ± 1.5 (0%)

Bone marrow buffy coat cells were treated with antibody and complement and cultured as described. Results are the mean ± SEM of colonies per 2 × 10^5 marrow cells for 2 experiments.

*Percent inhibition of colony formation.

†$p < 0.01$.

‡$0.05 > p > 0.025$.

Table 6. Reactivity of Monoclonal Antibodies L4F3 and L1B2 With BFU-E Determined by Indirect Immunofluorescent Staining and Fluorescence-Activated Cell Sorting

	BFU-E
Unseparated	54.0 ± 3.4
L4F3 positive	23.4 ± 11.4
L4F3 negative	122.2 ± 14.7
Unseparated	35.9 ± 3.0
L1B2 positive	2.8 ± 1.7
L1B2 negative	54.6 ± 17.6

Bone marrow cells were stained and separated by fluorescence-activated cell sorting as described (see Table 3). Cells from each of the sorted populations were cultured for BFU-E. Results are the mean ± SEM of colonies per 10^5 marrow cells for each population from two experiments in which each population was cultured in duplicate or triplicate.

cytic cells. L4F3 stained a slightly less mature myelocytic population in bone marrow, including a portion of the myeloblasts and the majority of cells up to and including metamyelocytes; and L1B2 detectably stained virtually all cells from the myeloblast to the myelocyte stage and only a portion of metamyelocytes. Finally, a determinant associated with monocytes, nucleated erythroid cells, platelets, and megakaryocytes, which was not found on granulocytes, was identified (5F1). None of these antigenic determinants was detected on thymocytes or lymphocytes, except T5A7, which reacted with a subset of PHA-activated peripheral blood lymphocytes. The reactivity of four of these antibodies with maturing granulocytic cells is schematically shown in Fig. 2.

Of particular importance in these studies was the extension of the characterization of monoclonal antibody reactivity to an examination of committed hematopoietic precursors. Of the committed stem cell populations examined, 1G10 reacted with most of the more mature CFU-GM and a smaller portion of the immature CFU-GM; T5A7 reacted with only a small portion of the more mature CFU-GM; and L4F3 and L1B2 reacted with most of the mature and immature CFU-GM (Fig. 3). In addition, antibody L4F3 reacted with a proportion of BFU-E. In contrast, the antibody 5F1 reacted selectively with CFU-E. Thus, we have identified a series of antibodies against maturation-linked determinants expressed by myeloid cells.

The monoclonal antibodies described in this article are potentially applicable to the study of a number of important biologic questions. First, they are potentially useful for depleting or enriching myeloid cell populations, including committed hematopoietic precursor cells. By separating populations of hematopoietic cells at varying stages of differentiation, it will be possible to precisely examine the interaction between cell populations in the regulation of hematopoiesis. Thus, 5F1 can be used to enrich CFU-E 12-fold and presumably by

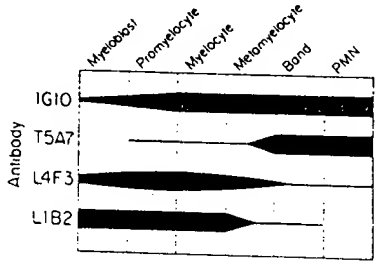


Fig. 2. Stages of granulocytic maturation. Summary of reactivity of monoclonal antibodies with granulocytic elements in bone marrow. The reactivity of each monoclonal antibody with granulocytic elements in bone marrow is schematically displayed. The thickness of each bar represents the percent of cells at each stage of maturation present in the sorted populations that were contained in the positively selected populations (see Table 1).

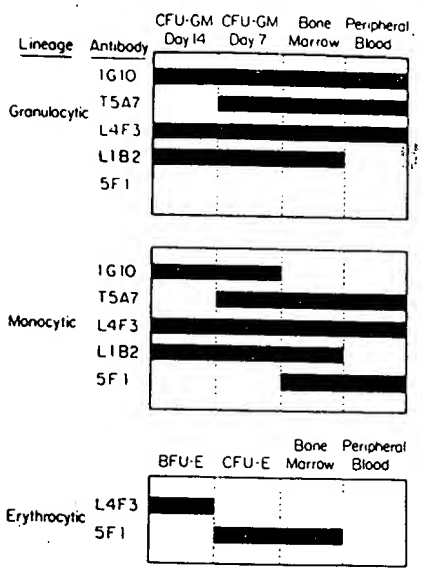


Fig. 3. Summary of reactivity of monoclonal antibodies with granulocytic, monocytic, and erythrocytic elements. A schema of the expression of myeloid associated antigens is shown. The reactivity of each monoclonal antibody with all or a portion of cells of each lineage within different maturation compartments of the hematopoietic system is shown. The solid bars are a nonquantitative representation of antibody reactivity with all or a portion of the cells in that compartment. The low fluorescent staining with 1G10 of a minor subset of peripheral blood and bone marrow monocytes is not shown. Reactivities of antibodies with cells in each compartment are based on results of antibody lysis experiments, as well as FACS analysis and fluorescence-activated cell sorting studies.

greater amounts when other antibodies, including those described here, are used to deplete other cells that express the 5F1 determinant. Further, 1G10, L4F3, and L1B2 can be used to select or deplete both mature and immature CFU-GM from bone marrow, while T5A7 will discriminate between these two CFU-GM populations. Since 1G10 is not reactive with circulating CFU-GM, it can also distinguish bone marrow from peripheral blood CFU-GM.[19]

Thus far, three monoclonal antibodies identifying myeloid-associated antigens have been reported to be reactive with stem cells. Specifically, two antibodies described by Linker-Israeli et al.[13] were reactive with monocytes and were shown to inhibit CFU-GM detectable following 10 days of culture. Hanjan et al.[21] described a single monoclonal antibody that inhibited CFU-GM detectable at day 15 of culture. Monoclonal antibodies with less restricted lineage expression also have been shown to react with committed hematopoietic progenitors. Specifically, monoclonal antibodies reactive with histocompatibility determinants (HLA-A,B; Ia-like),[25,26] blood group determinants (A, I, i),[27] determinants expressed by activated T cells,[28,29] and by leukocytes[30] have been shown to react with committed erythrocytic and myelocytic progeni-

tors. Certain of these antibodies have been used to separate populations of cells enriched for hematopoietic progenitors. Beverley et al.[30] used an antileukocyte monoclonal antibody, anti-HLE-1, to select committed erythroid and myeloid stem cells after eliminating mature myeloid elements from marrow with an antimyeloid monoclonal antibody, TG-1. They obtained 60–100-fold enrichment of both erythrocytic and myelocytic/monocytic progenitors in the same population. Griffin et al.[31] obtained up to 100-fold enrichment of CFU-GM from the peripheral blood of patients with chronic myelogenous leukemia (CML) using an anti-Ia monoclonal antibody after eliminating T and B cells and monocytes by immunorosetting techniques. It is not known whether erythroid precursors were also enriched in the selected populations. Similar strategies may now be evolved using antibodies reactive with more lineage-restricted antigens as well as less restricted antigens to isolate normal committed progenitor cells of a single lineage in highly enriched populations. Such techniques will be important for studies designed to dissect regulatory effects of purified hemopoietins on individual classes of hematopoietic progenitors.

In a second application, these antibodies provide probes to examine cell surface biochemical changes and eventually the responsible genetic alterations that occur during normal as well as malignant myeloid differentiation. To date, biochemical studies have suggested that the antigenic structures detected by each of the antibodies described here are expressed at least in part on glycolipids. We have recently shown that 1G10 recognizes the X-hapten carbohydrate determinant, which is expressed on both glycolipids and glycoproteins on the promyelocytic leukemia line HL-60.[32] Therefore, 1G10 detects a common haptenic determinant present on different molecular species in a single cell and is due to specific modifications of previously existing antigens. The importance of specific glycosylation events in altering cell surface antigenic structures during differentiation of myeloid as well as erythroid cells has been suggested by Hakomori et al.[33] This observation points out the need for caution in interpreting the molecular specificity of monoclonal antibodies, as they may detect epitopes found on multiple molecular species. Thus, biochemical studies to define the haptenic structures identified by monoclonal antibodies will be essential to our understanding of the relationship of the antibodies described here with those reactive with myeloid differentiation antigens generated in other laboratories.

In a third application, it will now be possible to relate the cell surface phenotype of malignant myeloid cells to stages of normal myelocytic differentiation based on the expression of maturation-linked antigens. Our experience to date has been that these antibodies react with the majority of myeloid leukemia cells tested, and they are useful in discriminating subsets of this disease. As previously reported,[18] the expression of these differentiation antigens is not necessarily consistent with the histochemical classification of leukemia. Thus, 5F1 may be expressed by myelocytic leukemias that positively stain with peroxidase but not with nonspecific esterase. Further, myelocytic leukemic cells may express differentiation antigens that are found on normal cells of nonmyelocytic lineages.[34] Eventual analysis of the genetic basis for this unexpected antigen expression may provide clues to the mechanism of transformation in myeloid malignancies.

Monoclonal antibodies reactive with differentiation antigens expressed by normal and malignant myeloid cells may be useful for treatment of nonlymphocytic leukemias, either for in vitro deletion of malignant cells from bone marrow in autologous bone marrow transplantation or for in vivo use as adjuvant therapy when tumor burden is at a minimum. The extent to which such antibodies are capable of distinguishing between leukemic cells and normal stem cells required for reconstitution of the bone marrow will determine their utility for purposes of therapy in ANL, and thus, at present, their usefulness remains an open question.

REFERENCES

1. Huber H, Polly MJ, Linscott WD, Fudenberg HH, Müller-Eberhard HJ: Human monocytes: Distinct receptor sites for the third component of complement and for immunoglobulin G. Science 162:1281, 1968
2. Barrett SG, Hansen KS, Bainton DF: Differentiation of cell surface receptors on normal human bone marrow myeloid precursors. Br J Haematol 48:491, 1981
3. Herborn HA, Valdimarsson H, Wickramasinghe SN: Development of human granulocyte and monocyte Fc receptors. Scand J Haematol 22:364, 1979
4. Burns GF, Cawley JC: Membrane receptors of human leukemic myeloid cells: Sequential expression of the Fc receptor. Br J Haematol 42:499, 1979
5. Ross GD, Jarowski CI, Rabellino EM, Winchester RJ: The sequential appearance of Ia-like antigen and two different complement receptors during the maturation of human neutrophils. J Exp Med 147:730, 1978
6. Winchester RJ, Ross GD, Jarowski CI, Wang CY, Halper J, Broxmeyer JE: Expression of Ia-like antigen molecules on human granulocytes during early stages of differentiation. Proc Natl Acad Sci USA 74:4012, 1977
7. Köhler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495, 1975
8. Breard J, Reinhertz EL, Kung PC, Goldstein G, Schlossman SF: A monoclonal antibody reactive with human peripheral blood monocytes. J Immunol 124:1943, 1979

9. Raff HV, Picker LJ, Stobo JD: Macrophage heterogeneity in man: A subpopulation of HLA-DR-bearing macrophages required for antigen-induced T cell activation also contains stimulators for autologous-reactive T cells. J Exp Med 152:581, 1980

10. Ugolini V, Nunez G, Smith RG, Stastny P, Capra JD: Initial characterization of monoclonal antibodies against human monocytes. Proc Natl Acad Sci USA 77:6764, 1980

11. Civin CI, Mirro J, Banquerigo ML: My-1, a new myeloid-specific antigen identified by a mouse monoclonal antibody. Blood 57:842, 1981

12. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 68:932, 1981

13. Linker-Israeli M, Billing RJ, Foon KA, Terasaki PI: Monoclonal antibodies reactive with acute myelogenous leukemia cells. J Immunol 127:2473, 1981

14. Majdic O, Liszka K, Lutz D, Knapp W: Myeloid differentiation antigen defined by a monoclonal antibody. Blood 58:1127, 1981

15. Perussia B, Trinchieri G, Lebman D, Jankiewicz J, Lange B, Roverra G: Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. Blood 59:382, 1981

16. Todd RF, Nadler LM, Schlossman SF: Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126:1435, 1981

17. Zola H, McNamara P, Thomas M, Smart IJ, Bradley J: The preparation and properties of monoclonal antibodies against human granulocyte membrane antigens. Br J Haematol 48:481, 1981

18. Bernstein ID, Andrews RG, Cohen SF, McMaster BE: Normal and malignant human myelocytic and monocytic cells identified by monoclonal antibodies. J Immunol 128:876, 1982

19. Andrews RG, Bernstein ID, Torok-Storb B: Monoclonal antibodies cytotoxic for leukemic cells: Reactivity with normal stem cells, in Mitchell MS, Oettgen HF (eds): Hybridomas in Cancer Diagnosis and Treatment. New York, Raven, 1982, p 147

20. Buckhardt JJ, Anderson WHK, Kearney JF, Cooper MD: Human blood monocytes and platelets share a cell surface component. Blood 60:767, 1982

21. Hanjan SNS, Kearney JF, Cooper MD: A monoclonal antibody (MMA) that identifies a differentiation antigen on human myelomonocytic cells. Clin Immunol Immunopathol 23:172, 1982

22. Kamoun M, Martin PJ, Kadin ME, Lum LG, Hansen JA: Human monocyte-histiocyte differentiation antigens defined by monoclonal antibodies: Identification of two distinct antigens shared by myeloid and erythroid cells. Clin Immunol Immunopathol (in press)

23. Schlunk T, Schleyer M: The influence of culture conditions on the production of colony-stimulating activity by human placenta. Exp Hematol 8:179, 1980

24. McLeod DL, Shreeve MM, Axelrad AA: Improved plasma culture system for production of erythrocytic colonies in vitro: Quantitative assay method for CFU-E. Blood 44:517, 1974

25. Fitchen JH, Ferrone S, Quaranta V, Molinaro GA, Cline MJ: Monoclonal antibodies to HLA-A,B and Ia-like antigens inhibit colony formation by human myeloid progenitor cells. J Immunol 125:2004, 1980

26. Robinson J, Sieff C, Delia D, Edwards PAW, Greaves M: Expression of cell-surface HLA-DR, HLA-ABC and glycophorin during erythroid differentiation. Nature 289:68, 1981

27. Sieff C, Bicknell D, Caine G, Robinson J, Lane G, Greaves MF: Changes in cell surface antigen expression during hemopoietic differentiation. Blood 60:703, 1982

28. Bacigalupo A, Podesta M, Frassoni F, Marmont M, Resso M, Giorgio C: A monoclonal antibody to antigens expressed on MLR-activated T cells inhibits granulocyte macrophage colony formation. J Immunol 127:2415, 1981

29. Bodger MP, Francis GE, Delia D, Granger SM, Janossy G: A monoclonal antibody specific for immature human hemopoietic cells and T lineage cells. J Immunol 127:2269, 1981

30. Beverley PCL, Linch D, Delia D: Isolation of human haematopoietic progenitor cells using monoclonal antibodies. Nature 287:332, 1980

31. Griffin JD, Beveridge RP, Schlossman SF: Isolation of myeloid progenitor cells from peripheral blood of chronic myelogenous leukemia patients. Blood 60:30, 1982

32. Urdal D, Brentnall TA, Bernstein ID, Hakomori S-I: A granulocyte reactive monoclonal antibody, 1G10, identifies the Gal β1-4 (Fuc α1-3) Glc NAc (X determinant) determinant expressed in HL-60 cells on both glycolipid and glycoprotein molecules. (submitted for publication)

33. Hakomori S-I: Glycosphingolipids in cellular interaction, differentiation and oncogenesis. Annu Rev Biochem 50:733, 1981

34. Smith LJ, McCulloch EA: Phenotypic classification of acute myeloblastic leukemia (AML) blasts: The presence in single cells of erythropoietic and granulocytic/monocytic markers. Blood 58(Suppl 1):153a, 1981 (abstr)

US005631348A

United States Patent [19]

Rosenblum et al.

[11] Patent Number: 5,631,348

[45] Date of Patent: May 20, 1997

[54] **PROTEIN SEQUENCE OF THE PLANT TOXIN GELONIN**

[75] Inventors: **Michael Rosenblum**, Houston, Tex.; **William J. Kohr**, San Mateo, Calif.; **Bharat Aggarwal**, Houston, Tex.

[73] Assignee: **The Research Development Foundation**, Carson City, Nev.

[21] Appl. No.: **254,662**

[22] Filed: **Jun. 6, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 119,899, Sep. 10, 1993, abandoned, which is a continuation of Ser. No. 908,959, Jul. 6, 1992, abandoned, which is a continuation of Ser. No. 567,220, Aug. 14, 1990, abandoned.

[51] **Int. Cl.**[6] **C07K 14/415**; C12N 15/29

[52] **U.S. Cl.** **530/370**; 536/23.6

[58] **Field of Search** 536/23.6; 530/370; 435/172.3, 252.3

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,446,240	5/1984	Nerenberg	436/542
4,810,648	3/1989	Stalker	435/191
4,888,415	12/1989	Lambert et al.	530/390
4,921,802	5/1990	Hall et al.	435/172.3

OTHER PUBLICATIONS

Lambert et al., *Cancer Treat. Res.*, vol. 37, 1988, pp. 175–209.

Ronneberger, *Dev. Biol. Stand.*, vol. 71, 1990, pp. 185–190.

Benoit et al., *Curr. Eye Res.*, vol. 9 Suppl., 1990, pp. 201–205.

Roselli et al., *J. Nuclear Med.*, vol. 30, 1989, pp. 672–682.

Dunbar, *Two-Dimensional Electrophoresis and Immunological Techniques*, 1987, Plenum Press, New York, pp. 32–34.

Stirpe et al., *J. Biol. Chem.* v. 255, 1980, pp. 6947–6953.

Jacobs, et al., *Nature* v. 313, 1985, pp. 806–810.

Falasca et al., *Biochem. J.* v. 207, 1982, pp. 505–509.

Sivam et al., *Cancer Res.* v. 47, 1987, pp. 3169–3173.

Young et al., *Science* v. 222, 1983, pp. 778–782.

Toy, J. L., *Clin. Exp. Immunol.*, 1983, 54, 1–13.

Primary Examiner—James S. Ketter

Attorney, Agent, or Firm—Benjamin Aaron Adler

[57] **ABSTRACT**

This invention relates to substantially purified gelonin, toxic fragments thereof, the DNA sequences encoding gelonin and use of the DNA for producing, by recombinant technology, gelonin, toxic fragments thereof and fusion proteins. More specifically, the invention relates to the primary amino acid sequence of gelonin, and of the DNA encoding said gelonin and the production of synthetic gelonin and toxic fragments thereof.

2 Claims, 13 Drawing Sheets

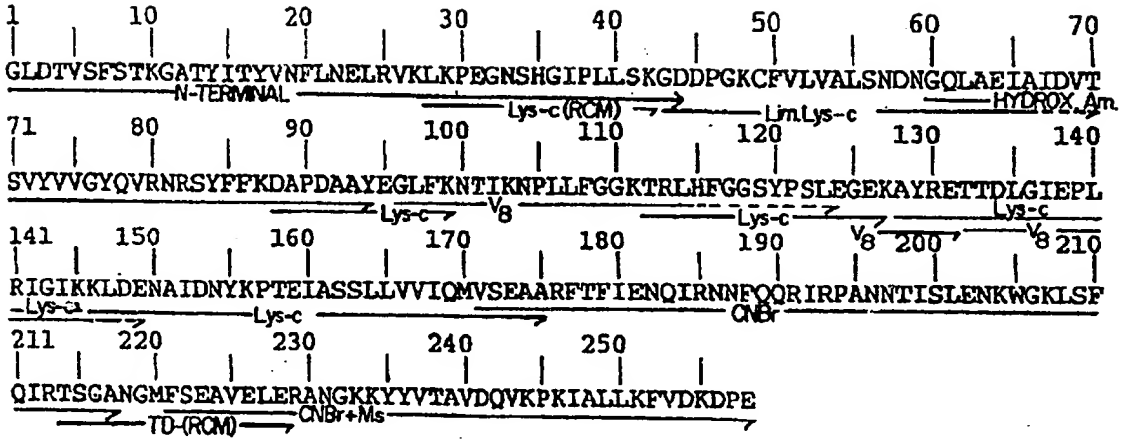

1 10 20 30 40 50 60 70
GLDTVSFSTKGATYITYVNFLNELRVKLKPEGNSHGIPLLSKGDDPGKCFVLVALSNDNGQLAEIAIDVT
N-TERMINAL
Lys-c(RCM)
Lm.Lys-c
HYDROX.Am.
71 80 90 100 110 120 130 140
SVYVVGYQVRNRSYFFKDAPDAAYEGLFKNTIKNPLLFGGKTRLHFGGSYPSLEGEKAYRETTDLGIEPL
Lys-c
V8
Lys-c
Lys-c
141 150 160 170 180 190 200 210
RIGIKKLDENAIDNYKPTEIASSLLVVIQMVSEAARFTFIENQIRNNFQQRIRPANNTISLENKWGKLSF
Lys-c
Lys-c
CNBr
211 220 230 240 250
QIRTSGANGMFSEAVELERANGKKYYVTAVDQVKPKIALLKFVDKDPE
TD-(RCM)
CNBr+Ms

FIG. 1

```
  1  GGNYTNGAYA CNGTNWSNTT YWSNACNAAR GGNGCNACNT AYATHACNTA
     CCNRANCTRT GNCANWSNAA RWSNTGNTTY CCNCGNTGNA TRTADTGNAT
     GlyLeuAspT hrValSerPh eSerThrLys GlyAlaThrT yrIleThrTy

                                                      ecoRI
 51  YGTNAAYTTY YTNAAYGARY TNMGNGTNAA RYTNAARCCN GARGGNAAYW
     RCANTTRAAR RANTTRCTYR ANKCNCANTT YRANTTYGGN CTYCCNTTRW
     rValAsnPhe LeuAsnGluL euArgValLy sLeuLysPro GluGlyAsnSer

          ecoRI
101  SNCAYGGNAT HCCNYTNYTN WSNAARGGNG AYGAYCCNGG NAARTGYTTY
     SNGTRCCNTA DGGNRANRAN WSNTTYCCNC TRCTRGGNCC NTTYACRAAR
       HisGlyIl eProLeuLeu SerLysGlyA spAspProGl yLysCysPhe

151  GTNYTNGTNG CNYTNWSNAA YGAYAAYGGN CARYTNGCNG ARATHGCNAT
     CANRANCANC GNRANWSNTT RCTRTTRCCN GTYRANCGNC TYTADCGNTA
     ValLeuValA laLeuSerAs nAspAsnGly GlnLeuAlaG luIleAlaIle

201  HGAYGTNACN WSNGTNTAYG TNGTNGGNTA YCARGTNMGN AAYMGNWSNT
     DCTRCANTGN WSNCANATRC ANCANCCNAT RGTYCANKCN TTRKCNWSNA
      AspValThr SerValTyrV alValGlyTy rGlnValArg AsnArgSerT

251  AYTTYTTYAA RGAYGCNCCN GAYGCNGCNT AYGARGGNYT NTTYAARAAY
     TRAARAARTT YCTRCGNGGN CTRCGNCGNA TRCTYCCNRA NAARTTYTTR
     yrPhePheLy sAspAlaPro AspAlaAlaT yrGluGlyLe uPheLysAsn

301  ACNATHAARA AYCCNYTNYT NTTYGGNGGN AARACNMGNY TNCAYTTYGG
     TGNTADTTYT TRGGNRANRA NAARCCNCCN TTYTGNKCNR ANGTRAARCC
     ThrIleLysA SnProLeuLe uPheGlyGly LysThrArgL euHisPheGl

351  NGGNWSNTAY CCNWSNYTNG ARGGNGARAA RGCNTAYMGN GARACNACNG
     NCCNWSNATR GGNWSNRANC TYCCNCTYTT YCGNATRKCN CTYTGNTGNC
     yGlySerTyr ProSerLeuG luGlyGluLy sAlaTyrArg GluThrThrAsp

401  AYYTNGGNAT HGARCCNYTN MGNATHGGNA THAARAARYT NGAYGARAAY
     TRRANCCNTA DCTYGGNRAN KCNTADCCNT ADTTYTTYRA NCTRCTYTTR
       LeuGlyIl eGluProLeu ArgIleGlyI leLysLysLe uAspGluAsn

451  GCNATHGAYA AYTAYAARCC NACNGARATH GCNWSNWSNY TNYTNGTNGT
     CGNTADCTRT TRATRTTYGG NTGNCTYTAD CGNWSNWSNR ANRANCANCA
     AlaIleAspA snTyrLysPr oThrGluIle AlaSerSerL euLeuValVal

501  NATHCARATG GTNWSNGARG CNGCNMGNTT YACNTTYATH GARAAYCARA
     NTADGTYTAC CANWSNCTYC GNCGNKCNAA RTGNAARTAD CTYTTRGTYT
      IleGlnMet ValSerGluA laAlaArgPh eThrPheIle GluAsnGlnI

551  THMGNAAYAA YTTYCARCAR MGNATHMGNC CNGCNAAYAA YACNATHWSN
     ADKCNTTRTT RAARGTYGTY KCNTADKCNG GNCGNTTRTT RTGNTADWSN
     leArgAsnAs nPheGlnGln ArgIleArgP roAlaAsnAs nThrIleSer

601  YTNGARAAYA ARTGGGGNAA RYTNWSNTTY CARATHMGNA CNWSNGGNGC
     RANCTYTTRT TYACCCCNTT YRANWSNAAR GTYTADKCNT GNWSNCCNCG
     LeuGluAsnL ysTrpGlyLy sLeuSerPhe GlnIleArgT hrSerGlyAl
```

FIG. 2A

```
651  NAAYGGNATG TTYWSNGARG CNGTNGARYT NGARMGNGCN AAYGGNAARA
     NTTRCCNTAC AARNSNCTYC GNCANCTYRA NCTYKCNCGN TTRCCNTTYT
     aAsnGlyMet PheSerGluA laValGluLe uGluArgAla AsnGlyLysLys

701  ARTAYTAYGT NACNGCNGTN GAYCARGTNA ARCCNAARAT HGCNYTNYTN
     TYATRATRCA NTGNCGNCAN CTRGTYCANT TYGGNTTYTA DCGNRANRAN
       TyrTyrVa lThrAlaVal AspGlnVaLL ysProLysIl eAlaLeuLeu

751  AARTTYGTNG AYAARGAYCC NGAR
     TTYAARCANC TRTTYCTRGG NCTY
     LysPheValA spLysAspPr oGlu

>length: 774

ecoRI(GAATTC)          96 107 572
not found
```

WHEREIN:

R = A,G	K = G,T	N = any
Y = C,T	M = A,C	S = C,G
B = C,G,T	V = A,C,G	: = unknown
D = A,G,T	W = A,T	- = ignored
H = A,C,T	X = unknown	

FIG. 2B

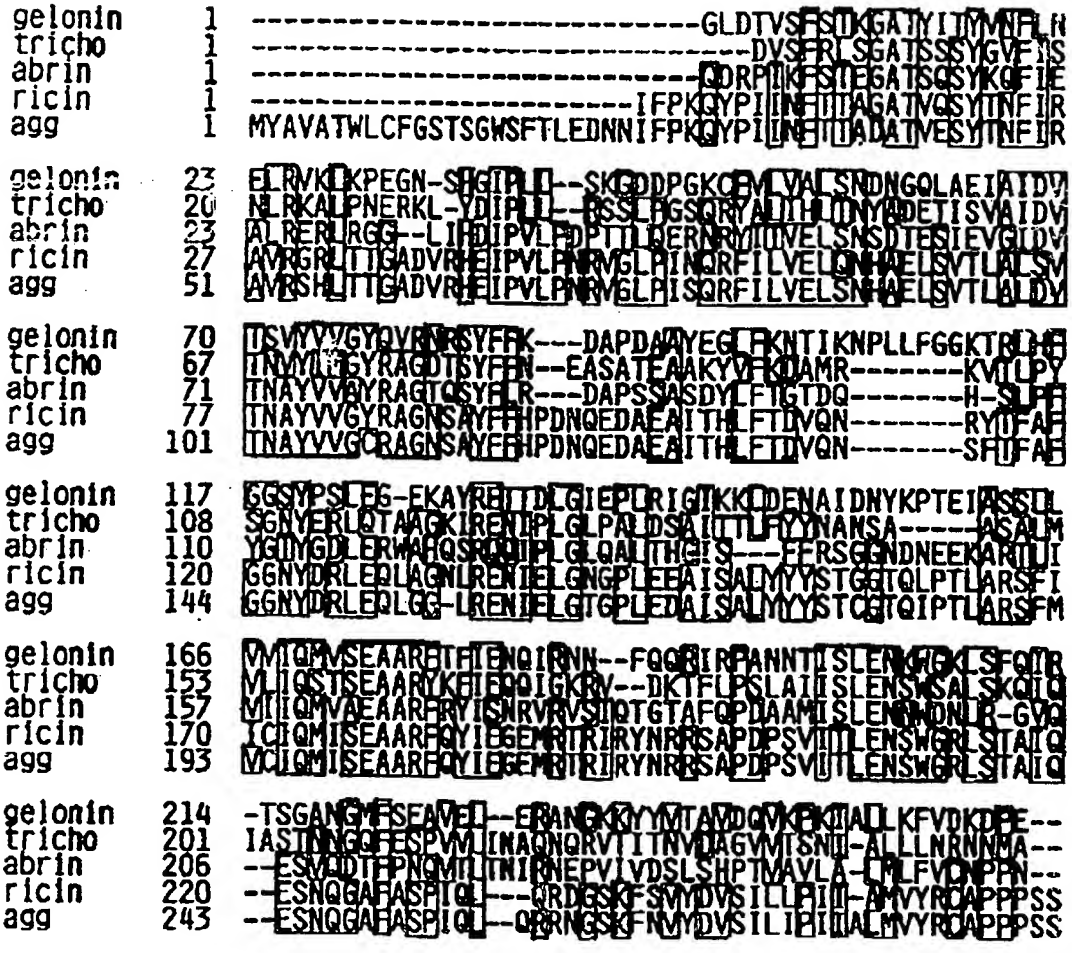


FIG. 3

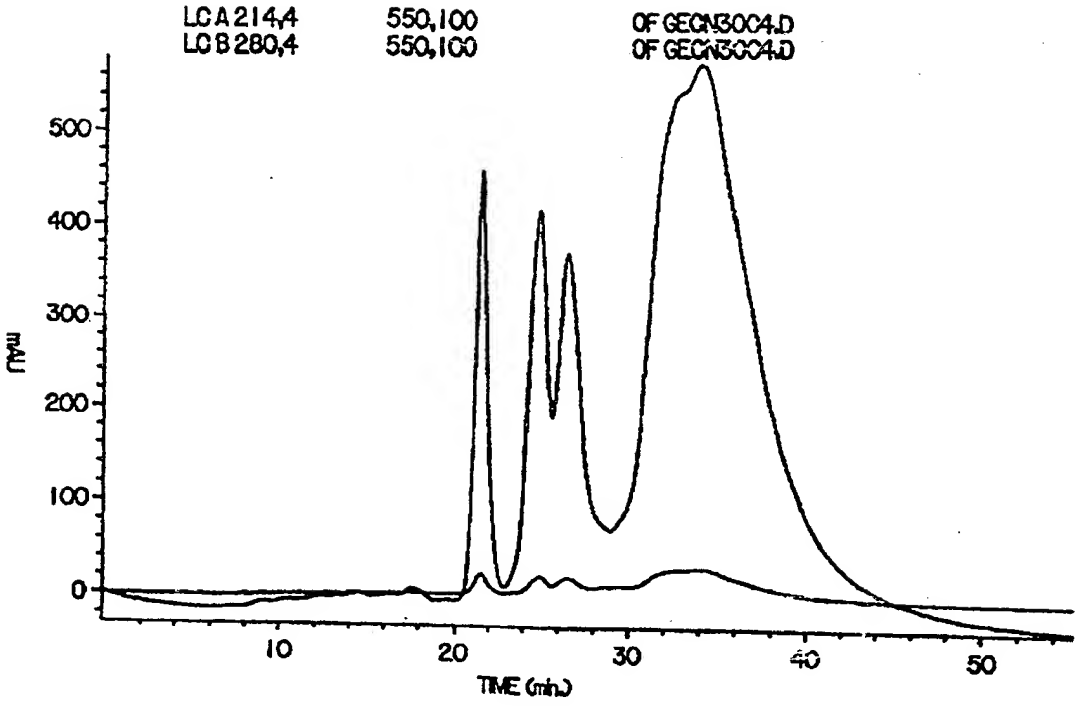

LCA 214,4
LCB 280,4
550,100
550,100
OF GEGN3004.D
OF GEGN3004.D
500
400
300
200
100
0
mAU
10
20
30
40
50
TIME (min)

FIG. 4

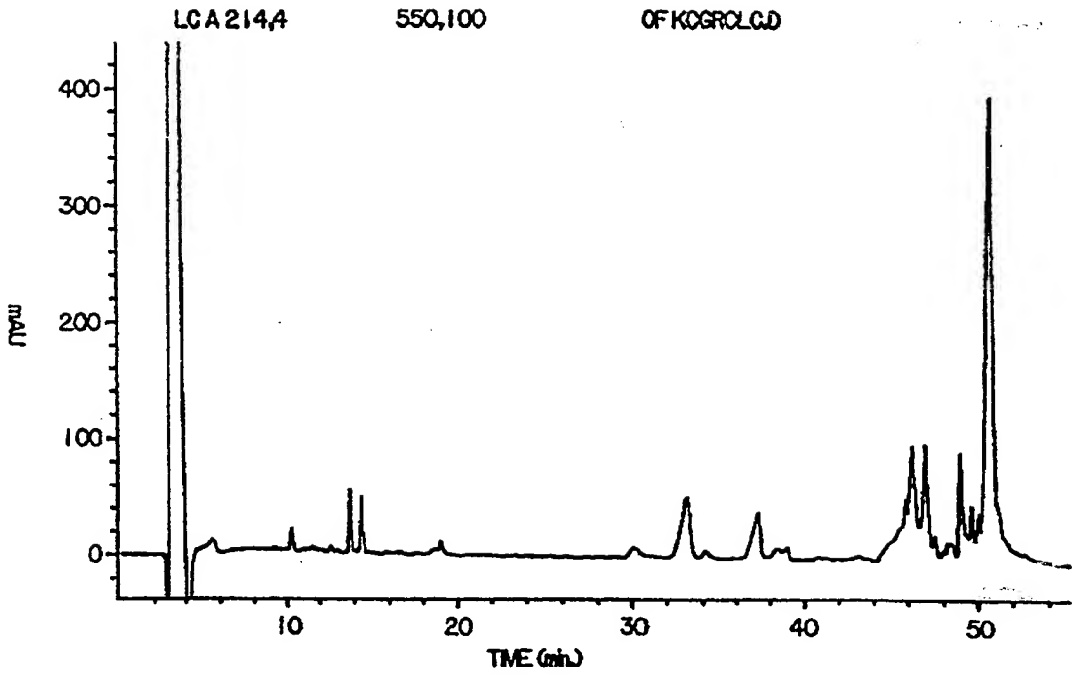

LCA214,4
550,100
OFKOGROLCD
400
300
200
100
0
mAU
10
20
30
40
50
TIME (min)

FIG. 5A

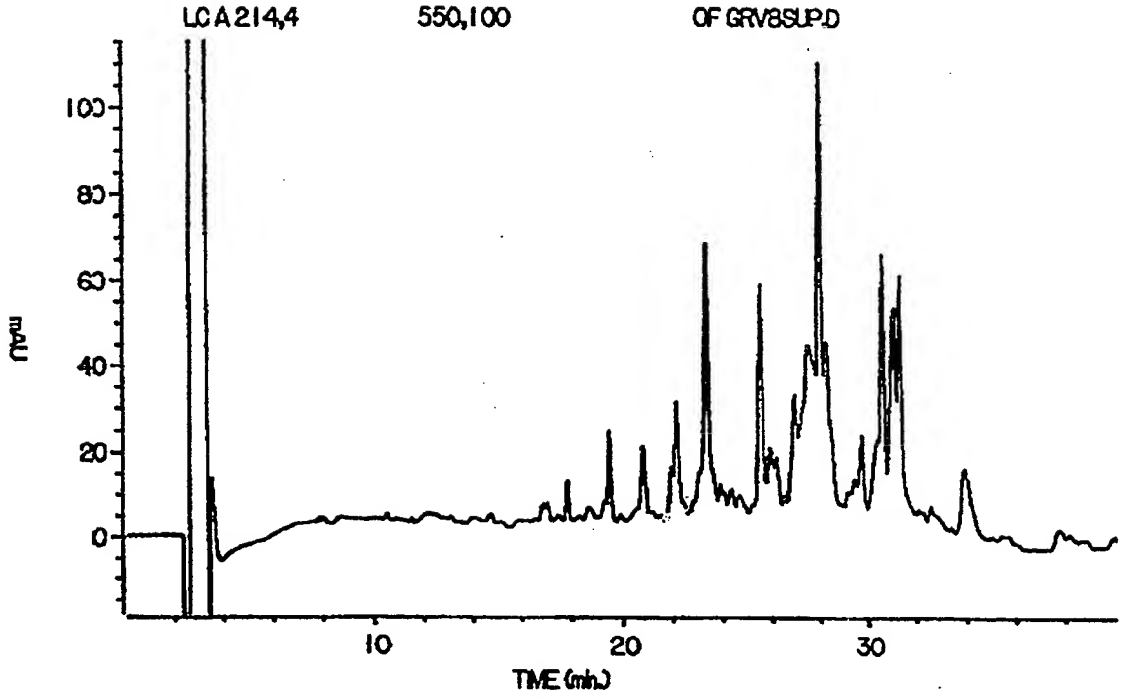

LCA214,4
550,100
OF GRV8SUP.D
100
80
60
40
20
0
mAU
10
20
30
TIME (min)

FIG. 5B

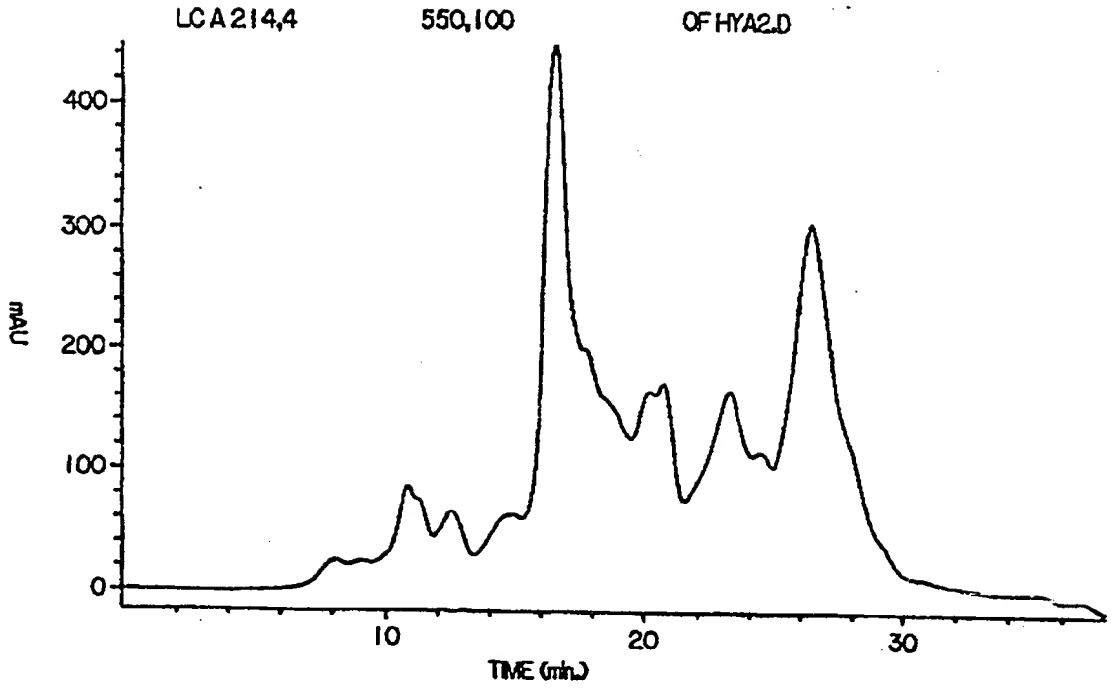


FIG. 5C

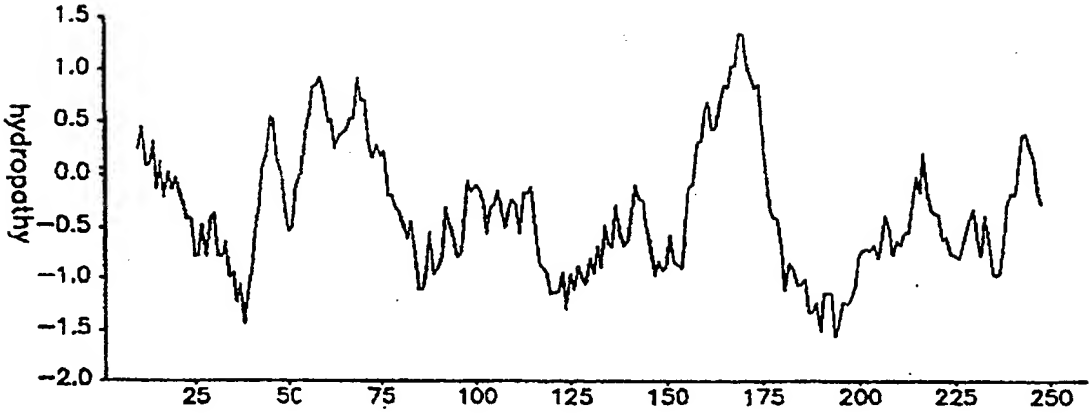

hydropathy
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
25
50
75
100
125
150
175
200
225
250

FIG. 6A

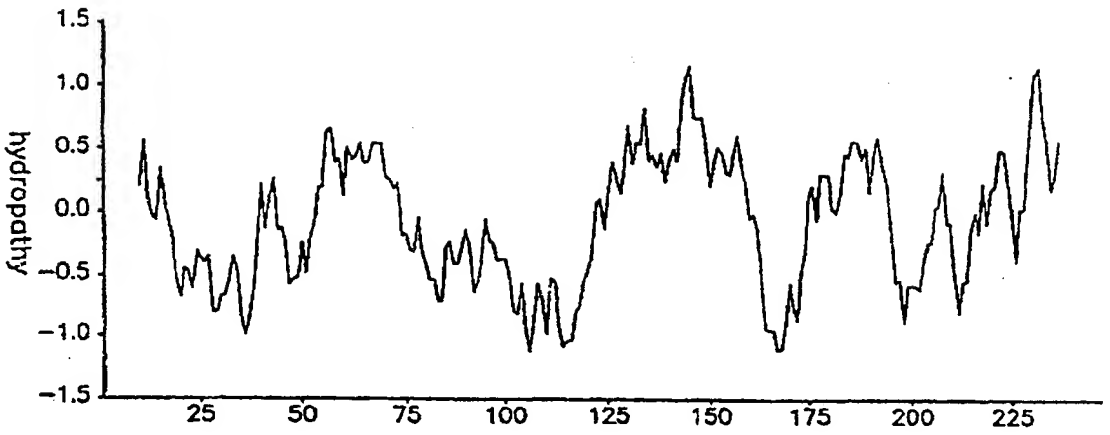


FIG. 6B

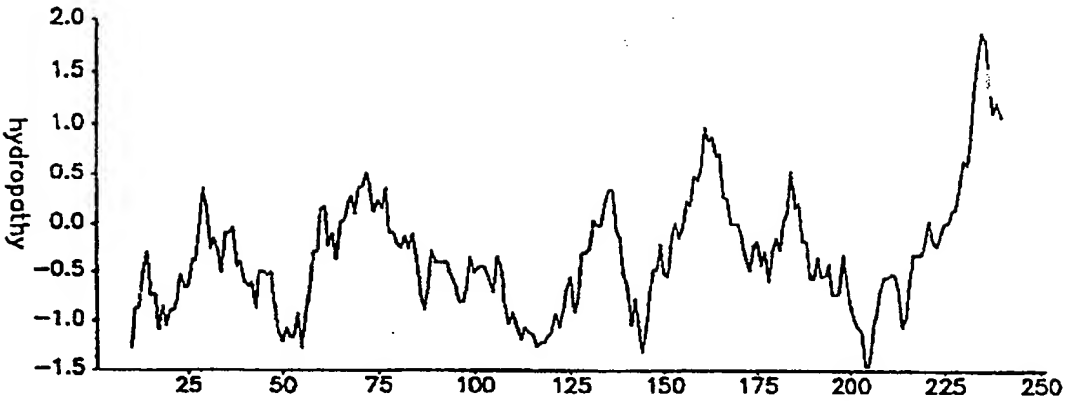

hydropathy
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
25
50
75
100
125
150
175
200
225
250

FIG. 6C

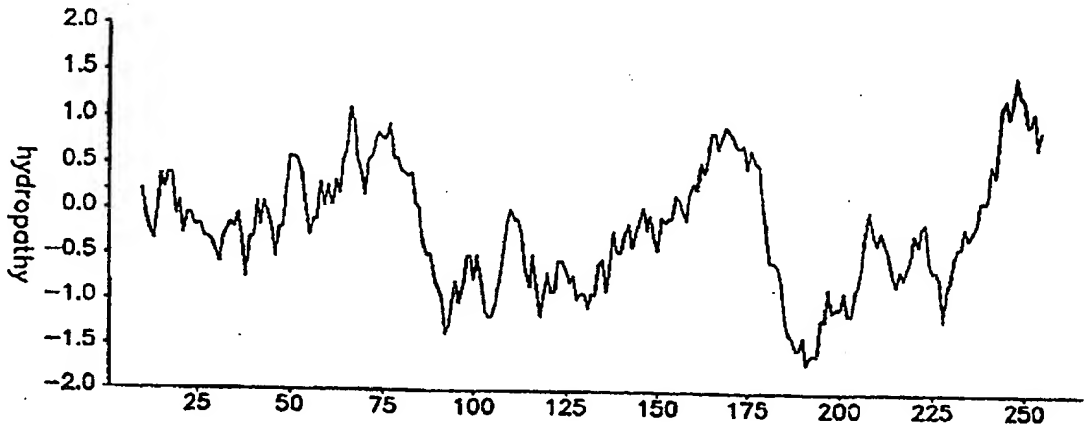

2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
hydropathy
25
50
75
100
125
150
175
200
225
250

FIG. 6D

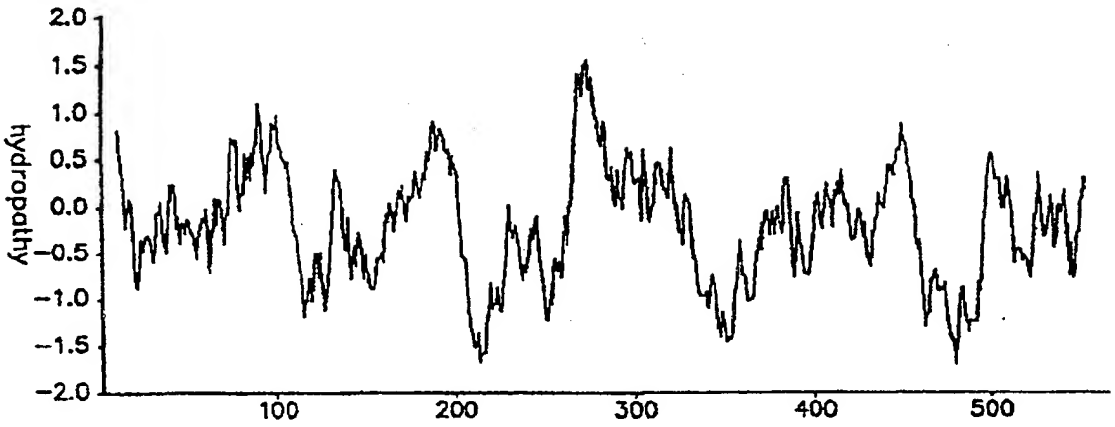


FIG. 6E

PROTEIN SEQUENCE OF THE PLANT TOXIN GELONIN

This is a continuation of application Ser. No. 08/119,899 filed on Sep. 10, 1993, now abandoned, which is a continuation of U.S. Ser. No. 07/908,959 filed Jul. 6, 1992, abandoned, which is a continuation of U.S. Ser. No. 07/567,220, filed Aug. 14, 1990, now abandoned.

TECHNICAL FIELD

This invention relates to substantially purified gelonin, toxic fragments thereof, the DNA sequences encoding gelonin and use of the DNA for producing, by recombinant technology, gelonin, toxic fragments thereof and fusion proteins. More specifically, the invention relates to the primary amino acid sequence of gelonin, and of the DNA encoding said gelonin and the production of synthetic gelonin and toxic fragments thereof.

BACKGROUND ART

A major challenge for the design of a drug for treatment of any disease is specificity and efficacy. Various drugs available for the treatment of cancer suffer from problems of this nature. The concept of targeting toxic drugs selectively to certain tumors has been a subject of intense research in the last few years (Thorpe (1985) *Biol Clin Applications* 84:475–512; Moller ed. (1982) *Immun. Rev.* 62:1–215). Recently both monoclonal and polyclonal antibodies, lectins, lymphokines and hormones which recognize specific determinants on the surface of the tumor cell have been used as carriers to deliver toxic agents into the cell, where the latter can exert their cytotoxic potential (Blattler, et al. (1985) *Biochemistry* 24:1517–1524; Frankel, et al. (1985) *J. Biol, Res, Modif,* 4:437–446; Reimann, et al. (1988) *J. Clin. Invest.* 82:129–138; Schwartz and Vale (1988) *Endocrinology* 122:1695–1700; Scott, et al. (1987) *J Natl. Cancer Inst.* 79:1163–1172; Singh, et al. (1989) *Biol. Chem.* 264:3089–3095; Srinivasan, et al. (1985) *FEBS Letters* 192:113; Schwartz, et al. (1987) *Endocrinology* 121:1454–1460). Toxic moieties thus far investigated with these delivery agents include radionuclides (Ghose, et al. (1967) *Br, Med, J.* 1:90–96), cytotoxic drugs commonly employed in cancer chemotherapy (Thorp and Ross (1982) *Immun. Rev,* 62:119–157; Deweger, et al. (1982) *Immun. Rev.* 62:29–45; Arnon and Sela (1982) *Immun. Rev.* 62:5–27; Pimm, et al. (1982) *Cancer Immun. Immunotherap,* 12:125–134; Rowland and Axton (1985) *Cancer Immun. Immunotherap.* 19:1–7) and proteins derived from bacteria and plants such as diptheria or ricin (Jansen, et al. (1982) *Immun. Rev.* 62:185–216; Raso (1982) *Immun. Rev.* 62:93–117. Vitetta, et al. (1982) *Immun. Rev,* 62:159–183; Nelville and Youle (1982) *Immun. Rev,* 92:47–73; Thorpe, et al. (1981) *Eur. J. Biochem.* 116:447–454). A specific molecule is designed by replacing the nonspecific B chain with an antibody or a hormone.

Bacterial and plant toxins, such as diphtheria toxin (DT), *Pseudomonas aeruginosa* toxin A, abrin, ricin, mistletoe, modeccin, and Shigella toxin, are potent cytocidal agents due to their ability to disrupt a critical cellular function. For instance, DT and ricin inhibit cellular protein synthesis by inactivation of elongation factor-2 and inactivation of ribosomal 60s subunits, respectively (*Bacterial Toxins and Cell Membranes*, Eds. Jelajaszewicz and Wadstrom (1978) Academic Press, p. 291). These toxins are extremely potent because they are enzymes and act catalytically rather than stoichiometrically. The molecules of these toxins are composed of an enzymatically active polypeptide chain or fragment, commonly called "A" chain or fragment, linked to one or more polypeptide chains or fragments, commonly

called "B" chains or fragments, that bind the molecule to the cell surface and enable the A chain to reach its site of action, e.g., the cytosol, and carry out its disruptive function. The act of gaining access to the cytosol is called variously "internalization", "intoxication", or "translocation". These protein toxins belong to a class bearing two chains referred to as A and B chains. The B chain has the ability to bind to almost all cells whereas the cytotoxic activity is exhibited by the A chain. It is believed that the A chain must be timely liberated from the B chain-frequently by reduction of a disulfide bond-in order to make the A chain functional. These natural toxins are generally not selective for a given cell or tissue type because their B chains recognize and bind to receptors that are present on a variety of cells.

The availability of a toxin molecule which is not cytotoxic to a variety of cells when administered alone has been limited. Utilizing certain naturally occurring single chain toxin molecules which do not themselves bind to cell surface receptors and, therefore, are not normally internalized by cells, has provided toxic molecules which are relatively non-toxic to most, if not all, cells when administered alone. Such naturally occurring single chain toxins known to date, include, but are not limited to, pokeweed antiviral protein (Ramakrishnan and Houston (1984) *Cancer Res.* 44:201–208), saponin (Thorpe, et al. (1985) *J. Natl, Cancer Inst.* 75:151–159), and gelonin (Stirpe, et al (1980) *J. Biol. Chem.* 255:6947–6953). These proteins are nontoxic to cells in the free form, but can inhibit protein synthesis once they gain entry into the cell. However, the availability of these single chain toxins in substantially pure form is limited due to the fact that they must be purified from plant sources in which they occur in relatively low amounts and the reproducibility of the concentration of the toxin in the plants is dependent upon plant growth conditions and plant harvest conditions.

Gelonin is a single chain polypeptide isolated from seeds of a plant, *Gelonium multiforum*, having a molecular weight of approximately 28,000–30,000 kd. Gelonin is a basic glycoprotein with an approximate isoelectric point of 8.15 and contains mannose and glucosamine residues (Falasca, et al. (1982) *Biochem J,* 207:505–509). In contrast to other plant and bacterial toxins, this protein is not toxic to cells by itself, but when delivered to cells through a carrier, it damages the 60s ribosomal subunit. In vivo and in vitro biological data suggest that gelonin is equivalent or superior to other plant toxins. In fact, the results of a comparison of gelonin conjugates in vitro and in vivo with other A chain conjugates indicated that gelonin had similar potency, better selectivity, better tumor localization, and more significant therapeutic effects (Sivan, et al (1987) *Cancer Res,* 47:3169–3173). However, the availability of a reproducible, readily accessible supply of gelonin from natural sources is limited. In addition, the purification of gelonin from plant sources is difficult and the yield is very low.

Gelonin by itself has been shown to be abortifacient in mice and enhances antibody dependent cell cytotoxicity (Yeung, et al (1988) *Internatl. J. Peptide Protein Res,* 31:265–268).

Several investigators have utilized gelonin as a cytotoxic agent chemically attached to monoclonal antibodies or to peptide hormone cellular targeting ligands. However, chemical modification of gelonin and cellular targeting moieties can reduce targeting efficiently and cytotoxic potential of gelonin itself. Furthermore, natural sources of gelonin are subject to variability in harvesting and plant growth which can affect gelonin cytotoxic activity. The ability to produce a synthetic gelonin toxin, chemically or utilizing recombinant technology, provides a plentiful, reproducible source of the toxin.

SUMMARY OF THE INVENTION

The present invention provides substantially pure gelonin having the amino acid sequence shown in FIG. 1. The present invention also provides the DNA sequence for gelonin shown in FIG. 2. Utilization of the sequences of the present invention to produce substantially pure gelonin in plentiful amounts by recombinant technology provides abundant amounts of the toxin which were not heretofore available from natural sources.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the amino acid sequence of gelonin.

FIG. 2 demonstrates the cDNA encoding for gelonin.

FIG. 3 demonstrates the homology of the gelonin amino acid sequence with the sequence of trichosanthin, Ricin A chain, Agglutinin precursor isolated from Castor bean and Abrin A chain.

FIG. 4 demonstrates the HPLC profile of CNBr fragments.

FIGS. 5A, 5B and 5C demonstrates the HPLC profile of (A) Lys-c, (B) Staphyloccus protease, and (C) Hydroxylamine digests of gelonin.

FIGS. 6A, 6B, 6C and 6D demonstrates the hydrophobicity plots of gelonin (A), trichosanthin (B), abrin (C), ricin (D), and agglutinin precursor (E).

DETAILED DESCRIPTION OF THE INVENTION

The term "substantially pure" when applied to the gelonin protein of the present invention means that the polypeptide is essentially free of other plant proteins normally associated with the gelonin in its natural state and exhibiting reproducible electrophoretic or chromatographic response, elution profiles, and toxic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the gelonin protein with other compounds.

Gelonin was purified from the seeds of the plant *Gilonin multiforum* by techniques known to those of skill in the art. The amino acid sequence was determined utilizing a modification of the Edman degradation method.

Samples of gelonin were applied to the reverse phase reaction chamber and subjected to Edman degradation. The N-terminal of gelonin was found to be heterogeneous (½ of the molecules of the protein were apparently one amino acid shorter than the others). This heterogeneity made it difficult to sequence much more than 40 cycles. Therefore, in order to determine further amino acids in the sequence, enzymatic cleavage was performed.

Internal sequence of proteins is generally obtained by digesting or cutting up the large protein molecule into smaller pieces with a combination of enzymes and chemical cleaveges. When native gelonin was exposed to various protolytic enzyzme digestions, it was found to be incompletely cleaved. This was found to be partly due to a disulfide bond in the N-terminal part of the molecule. Breaking of this bond by reduction and alkylation with iodoacetic acid yielded a fragment that was less soluable than the native material at the pH required for enzymatic digestion. A combination of digestion of native gelonin with trypsin, Lysine aminopeptidase (Lysc), staphlococcal protease (V8), and chymotrypsin yielded peptides mostly from the C-terminal portion of the molecule. This indicated that the N-terminal part of the molecule (from the N-terminal analysis to the Asp-Ala-Pro at residue 70) was not readily accessible by enzyme digestions.

Gelonin was cleaved with cyanogen bromide into 3 large peptides. Protein aliquots (0.2 mg/ml) were dissolved in 70% formic acid. A crystal of cyanogen bromide was added to the solution and the reaction allowed to proceed for at least 18 hours. The solution was then diluted with water and was applied to a small sequencing column. After sample application, a gradient of 1% to 10% n-propanol with 0.1% TFA was used to elute the protein fragmants. The elution profile is shown on FIG. 4.

Enzymatic digestion of the whole protein or of CNBr fragmants yeilded overlapping peptides. Enzymatic digestions with Lysyl endopeptidase in 0.1% SDS 100 mM Tris pH 8.0, Staphylococcus Aureus Protease in 0.1% SDS or trypsin in 0.1% Tween 20 were carried out. Gelonin contains one cysteine residue at position 49. Reduction and carboxymethylation yeilds a protein which recovers better on reverse phase HPLC and is more susceptible to enzymatic digestion. Therefore, most of the enzymatic digestions were carried out in 0.1% SDS or 0.1% Tween.

After the C-terminal 160 residues were aligned by a combination of CNBr digests and enzymatic cleaveges. The remaining unknown sequence between residues 40 to 70 was determined by a combination of chemical modification of cysteine with iodoacetic acid and solubization of the alkylated protein with SDS. The RCM alkylated gelonin was then cleaved with excess Lysc enzyme at 37° C. for short periods of time (1–5 hr.). The HPLC elution profile is shown on FIG. 5A.

This method yielded a new sequence that had not been seen before. This new sequence showed the existence of an Asn-Gly combination. This combination of amino acids is cleaveable by a chemical method using hydroxylamine.

Hydroxylamine cleavage was carried out by adding 100 ug of gelonin to freshly prepared hydroxylamine (2M) in 0.2 M Tris (pH 9.0) with 2M NaCl, mm EDTA and 10% ethanol. After incubation for 7 hours at room temperature, the entire reaction mixture was applied to a sequencing column. The colulmn was then washed with 1% TFA in water and either eluted with an acetonitrile gradient or was sequenced directly as a mixture. This chemical cleavege produced a large hydrophobic peptide that contained about a 200 amino acid sequence which connected with the Asp-Ala-Pro at residue 70. The elution profile is shown on FIG. 5C.

The remaining, short section of overlapping sequence from between residues 40 to 50 was determined by digesting gelonin without alkylation by Lysc in SDS. This digested away most of the C-terminal part of the material. Then this mixture was digested again by chymotrypsin. The products of this digestion were then separated by HPLC. Sequence analysis of a large pepticle revealed a sequence (SerThrLys) starting about 5 amino acids in from the N terminal end of the molecule. This was useful in that it removed the heterogeneous part of the molecule and allowed for a longer sequence run.

Gelonin protein comprises 258 amino acids, the sequence of which is demonstrated on FIG. 1. The amino acid sequence of gelonin was compared to other known sequences available in sequence data banks (Genbank, PIR, EMBL) to determine whether gelonin has any areas of homology with other proteins. Comparison of the gelonin amino acid sequence with other proteins having known amino acid sequences, demonstrated that the gelonin sequence is unique. Homology of certain portions of the gelonin sequence to portions of other proteins was detected. For instance, gelonin demonstrates a 36.0% homology with alphatrichosanthin from *Trichosanthin kirilowi,* 33.8% homology with Abrin A chain from Indian Licquorice, 35.2% homology with agglutinin precursor from Castor bean, 33.7% homology with Ricin D, A chain from Castor bean and 27.3% homology with antiviral protein (MAP) from *Mirabills jalapa.* A summary of the degree of homology to these and other proteins is shown on FIG. 3.

Hydrophobicity plots shown on FIG. 6A–6E demonstrate a similarity to hydrophobic regions of trichosanthin, Ricin and to other ribosomal inhibiting proteins.

A plot of the hydropathy of the gelonin structure shows a hydrophobic region in residues **35–80** and **150–180**. These are areas in which substantial folding of the molecule probably occurs. This similar hydrophobic pattern is also observed for other toxins (see FIGS. 6A–6E) and may suggest that the active enzymatic center may be contained within these folded regions. Therefore, the active enzymatic site may not be found in a linear region of the molecule and these structures may need to be adequately folded to attain the proper enzymatic center.

Utilizing the cDNA of gelonin, recombinant gelonin can be produced. Mutations can be specifically introduced into the molecule in order to provide recombinant gelonin lacking carbohydrate groups which can misdirect gelonin-antibody conjugates. Recombinant gelonin molecules can be produced by site directed mutagenesis to have greater toxic activity than the native molecule, to be more effectively internalized once bound to the cell surface by a carrier such as a monoclonal antibody or a targeting ligand such as IL-2, EGF, IFN, etc., to resist lysosomal degradation and thus be more stable and longer acting as a toxic moiety.

Recombinant gelonin molecules can also be engineered as fusion products to contain other functional modalities to kill cells such as an enzymatic activity, TNF, IFN activity, a second toxic activity, such as diptheria toxin action (wherein said second activity was through a different biological pathway than gelonin), thus creating a "supertoxin" or a toxin with multifunctional actions.

Fusion proteins can be engineered with gelonin to carry drugs such as chemotherapeutic agents or isotopes for radioimaging or radiotherapy. Gelonin peptides may have application as abortofacient agents, immuno suppressive agents, anticancer agents and as antiviral agents (such as an anti-HIV agent).

The following examples provide a detailed description of the preparation, characterization, and amino acid sequence of gelonin. The experimental methods utilized are described in detail in the examples below. These examples are not intended to limit the invention in any manner.

EXAMPLE 1

Purification and Characterization of Gelonin

Gelonin was isolated from the seeds of the plant *Gelonim multiforum* essentially according to the procedure as described (Stirpe, et al. (1980) *J. Biol, Chem* 255 6947–6953). Briefly, gelonin was extracted from the seeds by homogenization in buffered saline solution (pH 7.4). The supernatant was concentrated after dialysis against 5 mM sodium phosphate (pH 6.5) and the gelonin further purified by ion exchange chromatography as described below. The purity of the gelonin toxin was assessed by high pressure liquid chromatography (HPLC) and sodium dodecylsulphate-polyacylamide gel electrophoreseis (SDS-Page). Gelonin toxin migrated as a single band with an approximate molecular weight of 29–30,000 daltons.

Gelonin toxin activity was measured as described in Example 2 by protein synthesis inhibition in a cell-free system.

Seeds of *Gelonium multiflorum* were shelled and the nuts ground in a homogenizer with eight volumes of 0.14 M NaCl containing 5 mM sodium phosphate (pH 7.4). The homogenate was left overnight at 4° C. with continuous stirring, cooled on ice and centrifuged at 35,000 times g for 20 minutes at 0° C. The supernatant was removed, dialyzed against 5 mM sodium phosphate (pH 6.5) and concentrated using a pm10 filter. The sample was layered on a CM-52 ion-exchange column (20×1.5 cm) equilibrated with 5 mM sodium phosphate (pH 6.5). Material which bound to the ion exchange resin was eluted with 400 ml of 0 to 0.3 M linear NaCl gradient at a rate of 25 ml hour at 4° C. Five ml fractions were collected. The fractions were monitored at 280 nm in a spectrophotometer. The gelonin eluted in about fractions 55–70 and was the last major elution peak. These fractions were pooled, dialyzed against 0.1 M NaCl in 0.1 M Na_2HPO_4 buffer (pH 7.4). The sample was then applied to a Cibacron blue sepharose column (24×2 cm) previously equilibrated with 0.1 M Na_2HPO_4/0.1 M NaCl buffer. The column was washed with 3 column volumes of buffer and eluted with a 400 ml linear salt gradient (from 0.1 M NaCl to 2 M NaCl). Elution of the bound material was monitored by Lowry assay of the column fractions. The fractions containing the single protein peak were pooled and dialyzed overnight at 4° C. against PBS. Gelonin toxin was purified to greater than 97% purity as estimated from silver stained PAGE. The purity and the molecular weight of each preparation was checked on high pressure liquid chromotography using a TSK 3000 gel permeation column with 50 mM sodium phosphate buffer, pH 7.4 and 15% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-page). Gelonin migrated as a single band with an approximate molecular weight of 29–30,000 daltons.

EXAMPLE 2

Assay of Gelonin Activity

The gelonin activity was monitored in a cell-free protein synthesis inhibition assay. The cell-free protein synthesis inhibition assay was performed by sequentially adding to 50 ul rabbit reticulocyte lysate, thawed immediately before use, mixing after each addition, the following components: 0.5 ml of 0.2 M Tris HCl (pH 7.8), 8.9 ml of ethylene glycol, and 0.25 ml of 1 M HCl).

Twenty microliters of a salt-amino acid-energy mixture (SAEM) consisting of: 0.375 M KCl, 10 mM $Mg(CH_3CO_2)_2$, 15 mM glucose, 0.25–10 mM amino acids (excluding leucine), 5 mMATP, 1 mMGTP, 50 mMTris-HCl (pH 7.6), 10 ul Creatinine phosphate-creatinine phosphokinase, 8 ul [^{14}C] leucine (Amersham, 348 mCi/mmol), and adding 1.5 ul of solutions containing varying concentrations of the gelonin mixture. The mixture was incubated for 60 minutes at 30° C. ^{14}C-leucine incorporation was monitored in an aliquot of the mixture by precipitating synthesized protein on glass fiber filters, washing in 10% TCA and acetone, and monitoring the radioactivity in a Beta-counter using Aquasol scintillation fluid. Utilizing this assay, purified gelonin had a specific activity of 4×10^5U/mg protein. A unit of gelonin activity is the amount of gelonin protein which causes 50% inhibition of incorporation of [^{14}C] leucine into protein in the cell free assay.

EXAMPLE 3

Determination of Gelonin Amino Acid Sequence

The gelonin amino acid sequence was determined by the Edman degradation method using an automated amino acid sequencer as described in European Patent Application No. EP-257735. Large peptides and unfragmented protein were applied to the reverse phase portion of the sequence reaction chamber. Unwanted buffer components were washed off with excess water. The protein or peptide sample was then sequenced by Edman chemistry and the extracted ATZ amino acid derivatives were converted to the PTH form by 25% TFA in H_2O at 65° C. PTH samples were identified by reverse phase analytical separation on a Np 1090 column.

In order to obtain further amino acid sequence, the protein was digested with various proteolytic and chemical agents and then the peptides were purified by high performances liquid chromatography. Gelonin was found quite resistant to the exposure of trypsin (cleaves after arginine and lysine residues) and acetyl trypsin (cleaves only after lysine residue). The protein was found resistant to as much as 5% (w/w) of the enzyme. The resistance of gelonin to the proteolytic enzyme trypsin is not due to a lack of trypsin cleavage sites, since gelonin contains 21 lysine and 12 arginine residues. These results indicate that gelonin is perhaps a rigidly packed molecule which makes it inaccessible to proteolytic enzymes.

Since gelonin was found resistant to cleavage by proteolytic enzymes, chemical cleavage of the protein was examined.

EXAMPLE 4

CNBr Cleavage of Gelonin

Gelonin prepared as in Example 1 was dissolved in 70% formic acid. A crystal of cyanogen bromide was added to the solution. After at least 18 hours the solution was applied to either a small column (0.15 cm×5 cm) reverse phase (J. T. Baker; 15 cm C-1B bonded phase Cat II 7191-02) or analytical (4.6×100 mm) reversed phase column. A gradient elution of 1 to 70% n propanol with 1% TFA in water produce 5 peaks as shown on FIG. 6. Each of the peaks were sequenced and also used for further digestion by enzymes to piece together the entire sequence. Peak 1 was sequenced directly and gave a sequence starting with a Phe (F) that ran for 38 residues and ending with a Glu (E). This sequence was confirmed by mass spectroscopy and Lysc digestions of this isolated peptide. Peak 2 was sequenced directly and gave a sequence starting with a Val (V) that ran for 47 cy and was not interruptable after the ala at cy 47. Peak 3 was sequenced and gave the same sequence as peak 2. SDS gels of peaks 2 and 3 as well as Lysc digestion of peaks 2 and 3 showed that peak 3 contained the C-terminal CNBr peptide as well. Subsequent trypsin digestion of gelonin produced a peptide that connected these two CNBr peptide sequences. This trypsin peptide when sequenced gave the sequence TSGANGMFSEAVELER. Peak 4 and 5 both gave the N-terminal sequence GLDT . . . . This was used for some digestion by Lysc, ⅛, to give peptides from its C-terminal end.

EXAMPLE 5

Enzymatic Digestion of CNBr Cleaved Gelonin

Samples of whole protein or CNBr fragments were digested with Lysyl endopeptidase (Wako Chemical Dallas, Tex.) in 0.1% SDS 100 mmTris pH 8.0 or Staphylococcus Aureus Protease (Pierce) in.1% SDS or Trypsin (Sigma) in 0.1% Tween 20. Digestion mixtures were separated by HPLC and collected peptides were sequenced on the prototype sequence use gas-phase Edman sequencing methods.

EXAMPLE 6

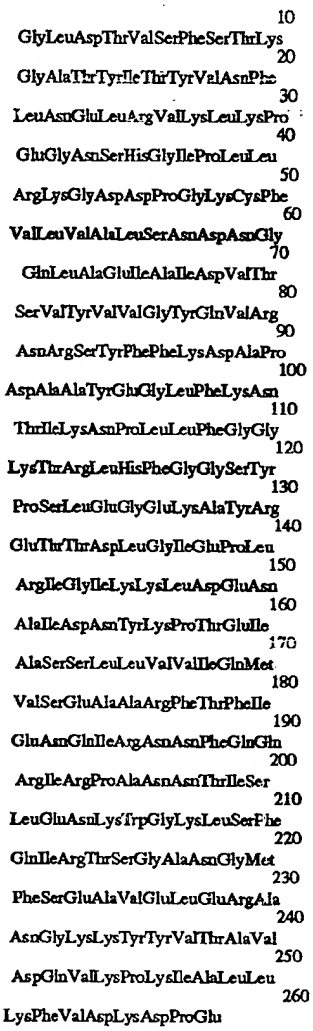
Amino Acid Sequence of Gelonin

A total of 258 amino acid residue sequences were obtained following analysis of the CNBr fragments obtained in Example 3. FIG. 1 shows the amino acid sequence of gelonin. Gelonin contains a total of approximately 258 amino acid residues. The DNA sequence was deduced from this amino acid sequence. The degenerate DNA sequence is shown on FIG. 2. Those skilled in the art will recognize that fragments and derivatives of either the gelonin amino acid sequence or the DNA sequence coding for gelonin may inhibit cellular protein synthesis but not bind to a cell surface receptor.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

What is claimed as new and is desired to be covered under Letters Patent is:

1. Substantially pure gelonin toxin having the amino acid sequence:



or a fragment or derivative thereof, said fragment or derivatives or having an activity which inhibits cellular protein synthesis but does not bind to a cell surface receptor.

2. A DNA sequence of the formula:

GGNYTNGAYA	CNGTNWSNTT	YWSNACNAAR	GGNGCNACNT	AYATHACNTA	YGTNAAYTTY	60
YTNAAYGARY	TNMGNGTNAA	RYTNAARCCN	GARGGNAAYW	SNCAYGGNAT	HCCNYTNYTN	120

-continued

MGNAARGGNG	AYGAYCCNGG	NAARTGYTTY	GTNYTNGTNG	CNYTNWSNAA	YGAYAAYGGN	180
CARYTNGCNG	ARATHGCNAT	HGAYGTNACN	WSNGTNTAYG	TNGTNGGNTA	YCARGTNMGN	240
AAYMGNWSNT	AYTTYTTYAA	RGAYGCNCCN	GAYGCNGCNT	AYGARGGNYT	NTTYAARAAY	300
ACNATHAARA	AYCCNYTNYT	NTTYGGNGGN	AARACNMGNY	TNCAYTTYGG	NGGNWSNTAY	360
CCNWSNYTNG	ARGGNGARAA	RGCNTAYMGN	GARACNACNG	AYYTNGGNAT	HGARCCNYTN	420
MGNATHGGNA	THAARAARYT	NGAYGARAAY	GCNATHGAYA	AYTAYAARCC	NACNGARATH	480
GCNWSNWSNY	TNYTNGTNGT	NATHCARATG	GTNWSNGARG	CNGCNMGNTT	YACNTTYATH	540
GARAAYCARA	THMGNAAYAA	YTTYCARCAR	MGNATHMGNC	CNGCNAAYAA	YACNATHWSN	600
YTNGARAAYA	ARTGGGGNAA	RYTNWSNTTY	CARATHMGNA	CNWSNGGNGC	NAAYGGNATG	660
TTYWSNGARG	CNGTNGARYT	NGARMGNGCN	AAYGGNAARA	ARTAYTAYGT	NACNGCNGTN	720
GAYCARGTNA	ARCCNAARAT	HGCNYTNYTN	AARTTYGTNG	AYAARGAYCC	NGAR	774

wherein

R = A or G
Y = C or T
B = C, G, or T
D = A, G, or T
K = G or T
M = A or C
V = A, C, or G
H = A, C, or T
N = any
S = C or G
W = A or T
X = unknown

or a fragment or derivative thereof, said fragment or derivative coding for gelonin or for a polypeptide having an activity which inhibits cellular protein synthesis but does not bind to a cell surface receptor.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,631,348 Page 1 of 5

DATED : MAY 20, 1997

INVENTOR(S) : Michael G. Rosenblum

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In Figure 1, amino acid residue number 41, "S", should read --R--.

In Figure 2A, amino acid residue number 41, "Ser", should read --Arg--.

In Figure 2A, nucleotides 121-122, "WS", should read --MG--.

In Figure 3, amino acid residue number 41 within the sequence "L--SKG" should read --L--RKG--.

In Column 1, line 34, "*Biol, Res, Modif,*" should read --*Biol. Res. Modif.*--.

In Column 1, line 41, "*Br, Med,*" should read --*Br. Med.*--.

In Column 1, line 50, "62:93-117. Vitetta" should read --62:93-117; Vitetta--.

In Column 1, line 50, "*Immun. Rev,*" should read --*Immuno. Rev.*--.

In Column 1, line 51, "*Immun. Rev*, 92" should read --*Immun. Rev.* 62--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,631,348

DATED : MAY 20, 1997

INVENTOR(S) : Michael G. Rosenblum

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In Column 3, line 60, "staphlococcal" should read --Staphylococcal--.

In Column 4, line 6, "fragmants" should read --fragments--.

In Column 4, line 9, "fragmants" should read --fragments--.

In Column 4, line 11, "Staphylococcus Aureus Protease" should read --*Staphylococcus aureus* protease--.

In Column 4, line18, "cleaveges. The" should read --cleavages, the--.

In Column 4, line 32, "NaCl, mm EDTA" should read --NaCl, 1 mm EDTA--.

In Column 4, line 35, "colulmn" should read --column--.

In Column 4, line 37, "cleavege" should read --cleavage--.

In Column 4, line 57, "sequences. demonstrated" should read --sequences demonstrated--.

In Column 4, line 62, "Licquorice" should read --Liquorice--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. :5,631,348

DATED :MAY 20, 1997

INVENTOR(S) :Michael G. Rosenblum

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In Column 5, line 46, "*Gelonim*" should read --*Gelonium*--.

In Column 5, line 48, "*J. Biol, Chem*" should read --*J. Biol. Chem.*--.

In Column 5, line 61, "*multiflorum*" should read --*multiforum*--.

In Column 6, line 20, "chromotography" should read --chromatography--.

In Column 6, line 40, "5mMATP, 1 mMGTP, 50 mMTris-HCl" should read --5 mM ATP, 1 mM GTP, 50 mM Tris-HCl--.

In Column 7, line 3, "performances" should read --performance--.

In Column 7, line 25, "reversed" should read --reverse--.

In Column 7, line 27, "produce" should read --produced--.

In Column 7, line 35, "interruptable after the ala" should read --interruptible after the Ala--.

In Column 7, line 52, "100 mmTris" should read --100 mM Tris--.